THE BIOCHEMICAL JOURNAL

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Edited for

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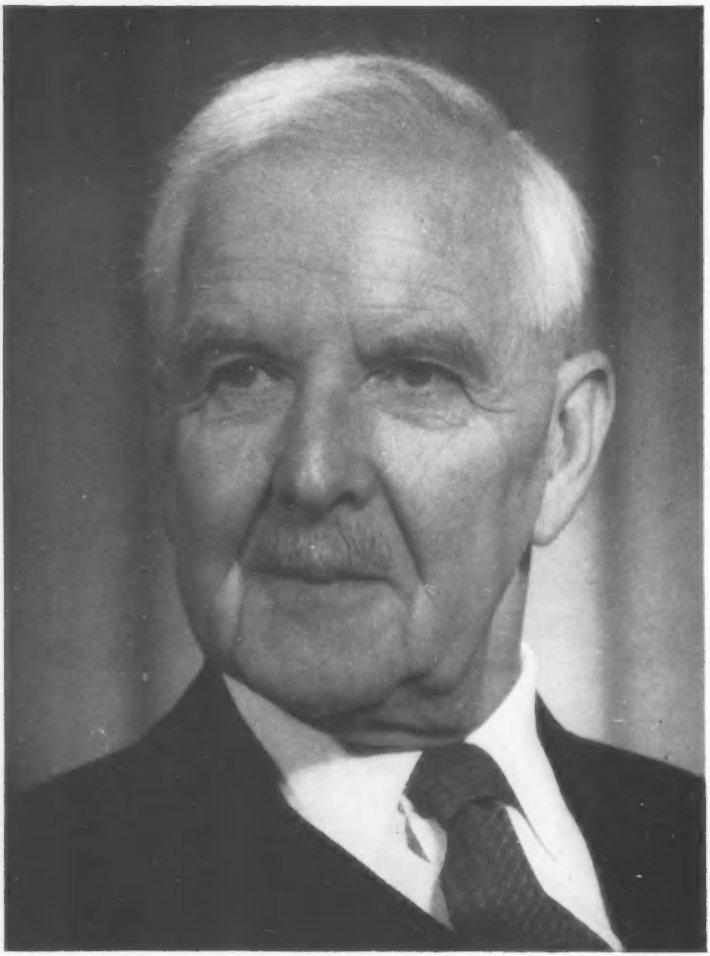

SIR RUDOLPH A. PETERS

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The faith of a Master in Biochemistry

By SIR RUDOLPH A. PETERS

THE FIRST HOPKINS MEMORIAL LECTURE

Delivered to a meeting of the Biochemical Society on 24 June 1958 in the Anatomy Lecture Theatre, Cambridge University

The most unusual honour and trust implicit in this invitation from the Biochemical Society to give the first Hopkins Memorial Lecture is one that can never occur to an individual again. It is tinged inevitably with the heavy responsibility of giving you some impressions of a unique mind and personality, and of the stretch of his scientific influence. Though other countries and other societies will have their heroes, to us British biochemists in the Biochemical Society Sir Frederick Gowland Hopkins will always be our Master biochemist, and it is fitting that his name and influence should be commemorated in a lecture. The explorer in any field must have some vision of what he wishes to do, and the faith to carry this out. Though it may seem paradoxical, in science such faith is necessary; but it must be justified by the realization of proof. Yet if time is to be saved in research, it is important to be a traveller on the right path, and it is particularly true in biochemistry, where the aim must be to understand in chemical terms what is going on in the cell.

In regard to the details of Hopkins's life, we all know that he attained many high honours; among them were the O.M., the Presidency of the Royal Society, the Copley Medal and the Nobel Prize, high distinctions indeed; but the emphasis to-day should lie upon his unique help to us as biochemists. How far did Hopkins have the vision of the way in which biochemistry should go, and how far was his faith sufficiently robust and unwavering to lead him and his pupils to continue in the right path?

The photograph (Plate 1) I find specially characteristic. It is in 1936, and shows Hopkins sniffing, for the first time I believe, the air of New Hampshire on the border of Canada (Quebec) and the U.S.A. Slight but wiry and energetic, though his appearance might well suggest a frailty, his performance belied this. Of his personal and intimate charm much has been written; it is well recorded in *Hopkins and Biochemistry*.* A particularly important though brief account of a very early Cambridge period is given by E. Mellanby, who was a pupil from 1902 to 1907; Lady Mellanby, Professor V. H. Mottram and Professor J. R. Marrack will remember something of this period. Though I started research with Barcroft, I can claim not only to have been on Hopkins's staff from 1919 to 1923, but to have come under his influence 50 years ago, when I came to Cambridge as an undergraduate. From 1910 I was taught by him for two years in the Part II Class, a very direct influence; at that time the subjects of physiology and biochemistry were not divided and we were brought into contact with a group of most original minds.

It was a wonderful experience for any young man to be in that old Cambridge Physiological laboratory during its heroic period, and by any, let alone modern standards, it was old. It occupied the quarters now devoted to the zoology museum, a natural history collection. Part of the laboratories was on the first floor, and another part in the famous cellar (see Plate 2). I think that the University should placard for visitors the steps to this cellar, where much of Hopkins's original nutritional work on rats jostled with that of Keith Lucas and Adrian on nerves, A. V. Hill on muscles, and a cave for the frogs. It reminds one of the Curies' shed, a triumph of determination over environment. In the class, what was remarkable to us as students was that Hopkins had always read the literature, and moreover would have an opinion about it, showing that he had 'appraised it' as he would say. We wondered how he had the time in the midst of his college work, where he even taught anatomy. I think he must have kept this up later, because Dr Marjory Stephenson once said to me that he was a kind of sponge for the literature. We all learnt much from his very individual Part II lectures.

It would be impossible to exaggerate the courtesy in a genuine humility and the patience with which he would consider points of view put to him by even quite junior students. Not always at his best in an elementary lecture, especially when given early, he would make the students his slaves afterwards in the practical class with his polite questions as to their opinions. This intimate respect of them as persons was one of his most engaging traits, and withal most educative—one had to think. This was how his time was rarely his own,

* *Hopkins & Biochemistry* 1861–1947. Cambridge: W. Heffer and Sons Ltd.

exemplifying the Chinese aphorism, 'the Sage has no self to call his own'. I find that I have been forced back to China for this expression, just as Dr and Mrs Needham were in their account! Many of us must have a picture still of the long queue waiting regularly outside his room, consisting as it did of folk from all sides of the University who brought to him their problems for his sympathetic advice. I still treasure a manuscript containing comments by Sir Frederick on a small memo which I wrote on muscle research. I treasure it not only for the shrewd remarks, but also because of an increasing gratitude and appreciation as the years go on of the valuable time he must have devoted to thinking about it. Though he had a core of merciless criticism in fact, he was otherwise so human that any stringency was forgotten. His Tea Club meetings exemplified so well his mastery of the subject, by his ability to put the speaker's remarks into perspective as well as by this kindly critique. He even carried a horror of hurting feelings to a fault, so that it was not always easy to get his real impression. He was indeed patient with his staff. The redoubtable 'Charles', to whom we all turned for help in the advanced class, once starved him of thiophen for some of his lactic acid tests, and he little protested. In fact, it subsequently transpired that there was a full bottle locked up in the cupboard! 'Charles' always felt it necessary to protect stores from the greed of research workers! Hopkins confessed to someone that he never knew what it was to be bored; but he got depressed when research went badly, and grudged the time spent on some of several committees, pointing out that people knew when a committee was cut but not when one cut research. Often his conversation started with the phrase 'worse luck', when he had not time to do something. He once said, a little petulantly for him, 'the truth is that no one has time for research after the age of 30'. I think that this is much less true nowadays with the increased recognition given to whole-time research.

H. C. Rümke, lecturing recently to the Dutch Academy, said: 'The creative man of science revealing things hitherto hidden is much closer to the creative artist than is commonly supposed.' May I expand this a little? In science the artistic is essential, but it is tempered art. The creative here consists in the revelation of what is already there by asking the right question and understanding the meaning of experiments. So far as there can never be perfection, scientific artists must always be at war with what seems to them to be their own inadequacies, and Hopkins was no exception to this rule.

With his assistant, E. J. Morgan, he was always at work, though only a small fraction of this reached publication; but it gave him a grip on the field and I never knew him not ready to stop and discuss some research result. Of course, a professor was less harassed by administration then, there was less money to spend! There were many delicious human touches. Even now I can see him skilfully avoiding the inconvenient visitor, dodging him with test tube in hand. Then there were the cigarette ends; 'Hoppy' seemed to require the stimulus of starting a cigarette to get his thoughts going; but, this accomplished, the cigarette was laid down and often forgotten, so that there might be several burning at once. It led to a kind of poker-work effect on the floor and even on the professorial desk, as Professor Young has pointed out. His wit was so pleasant too; when he had retired he made a small speech to the then newly founded Nutrition Society, saying that he was now on the shelf, and that this was an uncomfortable place from which to talk! Again, when his portrait was presented to him, he said that he had always wanted to have his portrait painted, but that as he went round College Halls he so often saw a wreck presented on the walls. His own portrait was not this. All these are personal points and they will strike others differently, yet they leave an indelible mark.

In regard to his research and teaching career, his value was recognized early by men such as Archibald Garrod and Michael Foster; but it should be stressed that, for various reasons, Hopkins hardly embarked on his real life's work until he was about 40, and to the end he felt that the fates had cheated him somewhat of time for research. Born in 1861, and numbering among the connexions of his forebears Thomas Gresham, he had an unusual schooling, and then had to escape from a business life in the City of London, as an insurance agent. Eventually, as an assistant at Guy's Hospital, he embarked in educating himself in medicine at the age of 28. Chequered as this early start was, it demonstrates that even in Victorian days there was room for the Hopkins's and Faradays to find their feet. I should not like to have to decide whether the early struggle toughened the later growth. It is interesting to compare this start with that of the famous French physiologist, Claude Bernard, who began in an apothecary's shop. For Hopkins, the Cambridge venture in 1898 was a big speculation and a great financial sacrifice; in this he was loyally supported by his wife, who was a very real partner and strength to him throughout. His professorship did not come until 1914, when his laboratory was located first in premises vacated by the Physiological laboratory, and later in 1919 partly in the Balfour Laboratory in Downing Place. The present Laboratory did not come to him until his sixty-fourth year.

SIR FREDERICK GOWLAND HOPKINS

SIR RUDOLPH A. PETERS—THE FAITH OF A MASTER IN BIOCHEMISTRY

(*Facing p.* 2)

The first floor belonged to the old Physiological Laboratory, which became part of the Biochemical Laboratory in 1914. Entrance to cellar below.

The steps to the cellar.

To put the biochemical contributions of Hopkins and his school into perspective, I find that I must give some kind of historical sketch of the development of biochemistry, which I attempt with a full realization of the limitations. We have to go back to Benjamin Moore, who was the first Professor of Biochemistry in England at Liverpool, and who gave some of the initial impulse to these studies as an independent discipline. We owe the *Biochemical Journal* to its foundation in 1906 by Moore and Whitley. Moore also contributed much to colloidal ideas of the cell and to knowledge of the influence of hydrogen-ion concentration, e.g. on the development of sea-urchin's eggs.

Before the Cambridge period, Hopkins's butterfly work and protein studies stamp him as a born investigator. His capacity for devising new chemical tools shows up later in his mercury agent for tryptophan, devised independently of Denigès. All of us who saw him at work will have vivid memories of a beautiful chemical technique as a manipulator; it was a joy to see him titrating. In the earlier nutritional work in Cambridge on proteins and then on vitamins, he exhibited as early as 1906 a remarkable intellectual grasp of the problems of accessory food factors as a whole and produced very convincing proofs of vitamins in his work on the rat published in 1912. We can say this without minimizing in any way the earlier Dutch contributions, the contemporaneous Norwegian and those in the U.S.A. During the first decade of the century, too, he formed the association with W. M. Fletcher which bore such fruit in the research on lactic acid in muscle, published in 1907. Indirectly, this led to Fletcher's pioneer work as the first Secretary of the Medical Research Council. Fletcher's work on carbon dioxide in muscle at the beginning of this century had already overturned the somewhat mystical 'inogen' theory, and their joint research on lactic acid gave point to the study of small molecules. If one assesses the position in 1910–12, it would not be true to say that no one had thought about enzymes in the cell. Hofmeister, for instance, had clearly good ideas of the place of biochemists. Leathes again had published his book on *Problems in Animal Metabolism*; Harden and Young had done their yeast studies, but the efforts were somewhat sporadic, and I do not think that there had been much effect upon the trend of physiological chemistry, which was still running mainly towards the study of excretory products, blood analysis and purely structural chemistry. (Perhaps parts of sugar metabolism should be excepted.)

It was at that point (in 1913) that we have Hopkins in his address to the British Association proposing that we should dare to peep boldly into the chemistry of the cell and follow the dynamic changes there taking place. It is this address which stamps him as the biochemical philosopher on whose thoughts we depend so much to-day. (I find it curious that at that time a young research worker like myself in that Laboratory did not realize that Hopkins had gone to Birmingham to give it.) Sherrington for one was much impressed; he spoke of the way in which Hopkins described an organized factory manifoldly hydrolysing, pulling to pieces and contemporaneously constructing and reconstructing; he felt himself privileged to see some of the microcosmic world in which the lecturer's thoughts took shape and did his bidding.

With the revelations of the electron microscope now at our disposal, it is hard for us to realize what a fight was needed to establish the position of intracellular enzymes. So few were then known. Even when we (Walker and I) in the 1920's tried to connect enzymes with vesication, there were very few to play with. The fight too has been long to establish the central point that chemical thought should be carried right into the cell. Even now it is not quite over. We can select a few passages from the 1913 address, though I think it should be read as a whole by biochemists. After stressing that studies of metabolism can be made simple, he says: 'I intend also to emphasize the fact that it is not alone with the separation and identification of products from the animal that our present studies deal, but with their reaction in the body, with the *dynamic* side of biochemical phenomena.' He emphasizes that the reactions in the cell, as influenced by enzymes, can be studied *in vitro*. 'We have arrived', he says, 'at a stage when it is logical to conclude that all metabolic tissue reactions are catalysed by enzymes, synthetic as well as in the opposite sense', and then we have the famous remark about the cell: 'its life is the expression of a particular dynamic equilibrium which obtains in a polyphasic system'.

Some touching-up of his views may now be necessary; but it is astonishing how close they come to the modern picture, and how right his ambition was to see these studies grow, even though he was heavily warned off the field when young by an important organic chemist who called it 'superchemistry'—and how right this was. Though he was apparently so pliant, when it came to the point of defending biochemistry, few could be stouter opponents. Physiology had much to do with launching biochemistry in this country, and at one time I thought him wrong to dissociate biochemistry and physiology; but as the years go on, I believe that it may have been a quite necessary stage in the emphasis of biochemistry as an independent philosophy to be carried on by those with the dual training of chemistry and biology. I was forced to such a view myself in relation to our

International Union. Yet here and there traces of the older attitude are still left. Even in 1939, there were many respected persons who could not believe that a tervalent arsenical might kill because it made a primary attack upon an enzyme system (in this case the pyruvate oxidase system). One respects criticism as the breath of life and scepticism; but there should be limits!

We may now pass to a review of some developments to see how these ideas worked out. In the nutrition field, there has been a steady development; again it was surprising how long it took to displace the idea that deficiency of some factor (vitamin or otherwise) could produce in itself pathological disturbance; so firmly rooted was the notion that disease must be produced by a positive reagent. I can remember arguments about this at a meeting in Cambridge in 1920. Yet Hopkins's ideas of the cell made the deficiency view simple.

In relation to the essential conception that intracellular enzymes were responsible for breakdown and synthesis of constituents of the cell, distinct phases can be traced in enzyme research. The first followed on the Pasteur–Liebig controversy; it starts with the Büchners' work on yeast juice and the attempted isolation of invertase by O'Sullivan and Thompson, say about 1896. Until the end of this time, that is until the early 1920's, we had an era when research was engaged virtually in determining various properties of enzymes then known and the limits for their action, as illustrated by research on the kinetics of invertase action. At the end of this period, there was the deliberate attempt of Willstätter and his colleagues to isolate enzymes, an attempt which gave much new technique; but it was left for the biochemist Sumner to prove the protein nature of an enzyme by crystallizing urease. During the early 1920's a step forward was taken by Meyerhof when he demonstrated so clearly that much of glycolysis did not need what had been called hitherto the architecture of the muscle; but that, with all the specificity involved, orderly reactions leading to lactic acid formation could take place in potassium chloride extracts of minced muscle, squeezed through muslin; these experiments played their part in Meyerhof's theory of the 'lactic acid' cycle. During this period too, from 1910–12 onwards, Warburg was studying oxidations; his work early proved that in sea-urchin eggs some oxidations were connected with structure, and later his slice technique opened a field of study wider than that of the tumours at which it was aimed. (It is interesting to note that Wigglesworth in Cambridge developed a slice technique independently.) Warburg became the forceful protagonist of the importance of iron in cell respiration, and in this was the settled opponent of Wieland's view that oxidation took place by transfer of hydrogen. As will be recalled in what is very familiar ground, Keilin largely reconciled these controversies with his scheme involving cytochrome *c* as the intermediate substance to be reduced through the agency of the dehydrogenases and reoxidized by the influence of oxygen through the cytochrome oxidase, the respiratory ferment of Warburg. In so doing, Keilin showed that cytochrome *c* was oxidized and reduced *in vivo* and produced our first sensible model of the respiratory chain. Warburg also studied his ferment *in vivo*.

Where did Hopkins and his philosophy fit into these developments? In my opinion, it was the thread which gave them coherence. The lactic acid work had laid the foundations of the glycolytic researches; these were also helped much by Embden's hexose phosphate isolation studies in muscle, following upon Harden and Young's classical research on yeast juice. A. V. Hill leant upon the lactic acid work in his studies of heat production in muscle. Above all, those in Hopkins's Laboratory at the time can remember the thrill of glutathione in 1921.

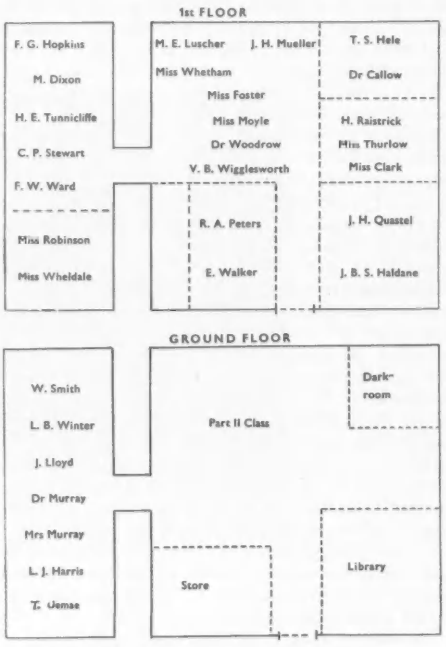

Fig. 1. Sketch plan of the Balfour Laboratory, during the years 1919–24, showing the approximate places in which various research persons worked.

It seems to be appropriate while we still recall it to give some picture of his colleagues in 1919–23 before the present laboratory was built. The research was done then in the old physiological laboratory and also the Balfour, now the music school. The latter was a converted chapel with a floor inserted at gallery level, which I had hurriedly partitioned for Hopkins in 1919 when acting as his adjutant. With the help of Dr Malcolm Dixon especially, I have constructed a plan giving the names of workers and the approximate places where they worked (Fig. 1). Dr Malcolm Dixon, as all will know, has been continuously behind much of the enzyme work in Cambridge since those early days, and it is particularly appropriate that he should be now President of the International Enzyme Commission. The upper floor was the large partitioned research room connected by a small bridge to Hopkins's laboratory (N. K. Adam worked in a small greenhouse). Below there was a small library and a store, and the advanced classroom where several distinguished people did their advanced class in biochemistry: Dixon, Roughton, J. Needham and Mrs Barbara Holmes, for instance*. There were still important researches going on in the old part of the laboratory, M. Stephenson, E. Gordon Young, Jordan Lloyd for part of the time (who kept her protein and colloidal work on gelatin going with clarity and enthusiasm), J. B. S. Haldane, R. A. McCance, R. Hill and R. Fearon. Hopkins's interest in nutrition was waning over this period, and Dr Malcolm Dixon remembers the day when he decided to swing the work towards oxidation. It is important to realize that in spite of the intense individuality of the persons making up this laboratory, Hopkins always maintained a very real intellectual control.

To return to glutathione, it is true that the chemistry took him seven years to clear up; but the impressive point was that a soluble substance with biochemical properties existed, able to react by addition and subtraction of hydrogen, as Wieland postulated. In a final assessment, it was a better proof than that of Wieland.

If there be any who wonder why the chemistry took so long, I would advise them to look at the original paper, where they will see how difficult the substance was to prepare, what pains were taken by Hopkins to get analyses done, and how closely they agreed with the suggestions of structure. Let them also think of the difficulties of isolating mixed disulphides, and remember that this had to be done without paper chromatography. It is indeed 'superchemistry'. Quite recently, Dr P. F. V. Ward in my Laboratory has gone through a similar experience in trying to purify a synthetic fluorocitric acid, which analysed well, but showed on a silicic acid column the presence of anything from 5 to 10 substances!

Some discoveries are made by logical inference. Several are nowadays the product of more than one mind, and this research may be expected to increase with the multiplication of special disciplines and techniques. But there will always be a place for the individual. Hopkins's discovery (with S. W. Cole) of tryptophan (I am told) was started by the awkward questions asked by a student in the advanced class. In the case of glutathione, Hopkins seems to have looked for acetoacetic acid in muscle with the nitroprusside reaction and come across the sulphydryl compound instead. The point of course was, and it is almost absurd to mention it, that he realized it was different. Most intelligent experiments are worth doing because they so often reveal the unexpected; but it is perhaps better to be not too much like the French physiologist Magendie, who is said to have made an idol of facts alone.

The scientific impact upon biochemistry of the isolation of this soluble sulphydryl substance was great and its significance continues to increase. It was the key which unlocked much of the work on intracellular enzymes, especially when coupled with the Thunberg dehydrogenase technique. Hopkins's efforts with pupils to find the 'fixed SH' in tissue preparations with which glutathione SH interacted, apparently unsuccessful at the time, contributed their quota to the present knowledge of SH enzymes. Professor Sidgwick once said (1928) that biochemists said their prayers to pH; perhaps he would now add SH, because SH as well as pH dominates biochemistry. So glutathione has become the ancestor of much research today. But to say this is not to detract from the brilliance of subsequent SH developments, which have been quite independent, and have involved much originality and new technique; our recent symposium on glutathione† is enough to demonstrate this. Nor should we be too complacent; even now can we say *in chemical terms* just why the SH in triose phosphate dehydrogenase is especially sensitive to iodoacetic acid and little so to a monosubstituted tervalent arsenical; or why the SH in

* An amusing story is told of these times. The workers on proteins and those trying to grow *Aspergillus* could not understand why the incubator seemed to do their specimens harm. Two of them therefore watched one night to see what happened. It was then noticed that one research worker came into the laboratory in the evening, hung his coat on the iron bar of the gas incubator, which accordingly responded with a large rise in temperature; when he left two hours later, he removed his coat and the incubator returned to normal; so by the next day the temperature was down to normal.

† *Glutathione*, Symposium No. 17, Biochemical Society. In the Press.

aconitase and *iso*citric dehydrogenase is insensitive to iodoacetic acid, and very easily attacked by disubstituted tervalent arsenicals?

In the 1920–30 decade we can notice that, following the work on glutathione, much effort in the Cambridge Laboratory was devoted with the dye technique to the reactions of dehydrogenases. In work on xanthine oxidase, for instance, Hopkins pushed forward such researches and encouraged oxidation–reduction studies at the microscopic level. I am afraid that these studies left many physiologists, and others, little impressed with the possible impact of the great gains in the differentiation of specific intracellular enzymes, though their attention did soon focus upon the enzyme cholinesterase. The studies of Quastel and colleagues on succinic dehydrogenase and its inhibition by malonate were a first example of competitive inhibition, and in this may be reckoned as the precursor of antimetabolite studies. It was a moment of great excitement for us in Oxford when we found about 1930 an effect of vitamin B_1 (thiamine) *in vitro*, because this led to the connexion of intracellular studies with pyruvic acid, with vitamins and cellular oxidation; and it definitely related this biochemical lesion with pathological changes. These researches, I really think, sealed the connexion of the intracellular enzyme with physiological events, oxidations and vitamins, and we were fortunate that a prolonged research should have matured in this way. Other B vitamins later took their place in coenzymes. At this time (1930) too, the earlier background research on parts of the tricarboxylic acid cycle was in progress by Szent-Györgyi and others to lead ultimately to the formulation by Krebs of his citric acid cycle. In the later 1930's, the progress of research in the glycolytic field led to the independent formulation of coupled phosphorylation by D. M. Needham and Meyerhof with their colleagues. Arising from this we have come to the realization that adenosine triphosphate is a special kind of fuel and of the special position of oxidative phosphorylation; at present the biochemical fire is blazing in so many places that it is now difficult to discover the Hopkins thread.

Such a short sketch is a dangerous over-simplification; it leaves out so many distinguished persons who worked in the present laboratories and important fields like the steroids; still it may suffice to indicate how justified Hopkins was in his address to the British Association in professing his faith in the value of a ruthless exploration of specific intracellular enzymes and in studies of the life of the cell carried out on small molecules with the firm belief that the logic of chemistry could be used in interpreting biochemical events.

From a perusal of his writings over this period, though he had stressed the colloidal nature of his 'dynamic equilibrium' in his address, he seems to have travelled more to the idea that the specific enzyme was all important in the control of intracellular events. Here he may have been influenced by the organization of enzymic reactions in muscle minces, which we now know would still contain much organization at the level of mitochondria and microsomes. Doubtless he felt rightly (as did many others) that accurate knowledge of events in cells must wait upon a deeper and more intensive study of enzymes in isolation, and that the wisest policy was to encourage this as a sound basis for reconstruction. He was certainly much surprised, as I know from a letter, when Lundsgaard showed that iodoacetic acid could produce a muscle which contracted without formation of lactic acid; even now do we really know how to relate the biochemistry to the physiological events of contraction? It is hard to know whether in his inspired phrase 'dynamic equilibrium' he really pictured the extraordinarily rapid interchanges of which we are now aware through the use of radioactive isotopes. At any rate, it was the inspired guess of genius.

In parenthesis, I might mention a personal impact. Having thought that I knew something, after several years' work, about the behaviour of pyruvic acid in the old-fashioned brain brei, I was truly astonished when working with Professor and Mrs Chain in Rome last summer to find the extent of the reactions in which the ^{14}C from labelled pyruvic acid had taken part. In a two-way paper chromatogram as scanned by their two-dimensional scanner, the substances labelled were hexose phosphate esters, phosphoglycerate, citrate, α-oxoglutarate, glutamate, alanine, aminobutyrate and some other unidentified substances. The Chains and colleagues have already published work of a similar type starting from glucose.

D. D. Woods pointed out, in his Memorial Lecture on Marjory Stephenson, that she stressed in relation to microbiological studies the different levels of organization in bacterial cells, all of which could under appropriate conditions be worth while objects of research. In their enthusiasm, I think that some biochemists have claimed too much for their isolated studies; this Hopkins would not have done, because with his background and intellectual equipment he never forgot the importance of the biological outlook—it was part of him. No doubt in his efforts to teach that the inside of the cell was within reach of the experimentalist, he was led to overstress the small molecule. It is hard to know what would be meant now by the colloidal system of coexistent phases, because colloidal science has much changed. Some of us, nevertheless, in the 1930's did begin to put forward views that the cell was even more organized

chemically than previously supposed; perhaps we could say shortly that it had a real geography. In essence, this amounted to saying that the same mode of thought should be applied to the cell as one applies to the carbon atoms in a benzene molecule. In my own case, these attempts at conscious co-ordination arose especially from the Hardy–Langmuir conceptions of surface orientation of molecules, which gave substance to the idea that even a monomolecular film of a fatty acid could behave on one side as a dissociable acid and on the other as virtually a paraffin. Such ideas really transformed some aspects of colloid chemistry, and it further became known that the nature of a metal surface could influence chemical changes taking place near it. It seemed that there could be an organization on these lines inside the cell, taking part in the co-ordination of cell reactions. This is not the place to elaborate such views, which I regarded as an extension of dynamic biochemistry; but they involved more emphasis upon the geography than Hopkins liked—I think probably because he was alarmed that such thoughts harked back to the mystical and entirely sterile idea of the 'inogen molecule'. But in his later Linacre lecture he warned of 'organization of an undreamt of complication'. Where I think that the views differed was in the suggestion that the enzymes of the cell themselves might be under the control of some mosaical structures. I feel sure that Sir Frederick would have been fascinated to follow the electron micrographs of mitochondria and microsomes and the later suggestions that some of these may be fragments from some larger sheaths. The remarkable thing is that they can be studied at all.

Dr de Duve and his colleagues, and others, have been carrying these studies further. In my Laboratory Dr V. P. Whittaker has recently collaborated with Dr K. M. Smith of the Molteno Institute to find that the particles which have the cholinesterase and acetylcholine, and which Miss Hebb has shown to contain the choline-acetylase, separate from the mitochondria in the Spinco centrifuge, and therefore are separate from the enzymes of the tricarboxylic acid cycle. Evidently we have some way to go in unravelling these complications.

Now we see in progress from several sides the postulation of very long molecules of nucleic acid to act as templates for the genes. Dr E. F. Gale studies such problems in bacteria. At the same time, the structure of a protein in terms of the order of its constituent amino acids has become revealed for the first time in Hopkins's old laboratory by Sanger, where Professor Chibnall turned especial attention to studies of the proteins, and where the present Professor actively encourages studies on the structure as well as the function of some of the hormones. It is interesting too that two pupils of this Department (I refer to Martin and Synge) devised paper chromatography, which played a prominent part in the elucidation of the structure of the protein, and is so extensively used now that our Society meetings are often leopard-like! The wheel has come full circle; the giant molecule is having its revenge, but it is a Pyrrhic victory; for we have retained enough of Hopkins's philosophy to realize that it is not too mysterious to study in chemical terms. Perhaps we have become too courageous!

Our more purely chemical friends are often puzzled when we say that biochemistry is a separate discipline; they see us using essentially chemical techniques in our work, even if we often have to shape our own tools. Wherein then is the difference? It is mainly in the mental attitude; in the fact that, even though we have to concentrate upon isolations, we should have eyes all the time upon the application of chemical facts in life. Subsidiarily, however, there is the strangeness of the chemical medium. So much takes place at pH 7·0, and Nature in the course of evolution seems to have learnt more than any chemist yet knows about chemistry at this pH and comparatively low temperatures; further, organisms have learnt to defend their pH as part of their internal environment. Our respiratory centre is sensitive to a change of about 0·01 pH. In 1919 I remember that in the advanced class we looked at some vinegar eels which had been brought to me by a zoologist from one of the College High Tables. We were amazed to find that these beasts put up with a concentration of some 0·01N-hydrochloric acid. This fired Hopkins, who was disappointed that the class, with their narrower range of knowledge, was not so excited. Incidentally, this organism also stands very deep freezing. I believe that we ought to think more about pH even than we do. Many years ago, I proved that the apparent pH at the interface between an aqueous and lipoid phase was shifted as compared with the bulk pH, and I still feel that some of the mysteries raised by pH optima for some enzymes far removed from the pH of the cell are to be explained in this area. We also know that buffers can alter the pH optima of isolated enzymes in solution.

Quite recently, Dr R. M. C. Dawson in my Laboratory has found a relevant phenomenon in connexion with his studies on phospholipids. It has been recognized for some time that two steps were needed to take off the two fatty acids in lecithin (or kephalin) by enzymes known as phospholipase *A* and *B*. Working with a phospholipase *B* preparation from *Penicillium notatum*, Dawson found that activation of this for phospholipase *A*

activity could be induced by adding to the substrate various acidic lipids, one of which is cardiolipin. The net effect is to convert one enzyme activity into two. This action appears to lie in the surface field as the activation goes parallel with decreasing lipid solubility of the lecithin and an increase of hydrophilic properties. With Dr A. D. Bangham, of the Pathology Department, this effect has been traced to a change of the electrical charge of the substrate particles from positive to negative. This then gives a new and concrete example of how surface chemistry may alter conditions in cells, and is clearly of much theoretical interest. Much more knowledge is wanted in the surface field, even on the technical level, as a basis for the technique of chromatographic column separations.

There are still important gaps in our knowledge of the chemistry of these microheterogeneous systems. Taking pH as an illustration, a simple calculation which I made years ago shows that, at the neutral point, particles like the mitochondria or the virus even will have on average much less than one dissociated hydrogen ion. I know that the thermodynamic argument is that there will be a field for a fraction of the time; but I find this rather unreal. The explanation seems to involve some unknown point, which may be connected with the speed of adjustment of this type of equilibrium, or it may even be that Nature takes advantage of an aqueous background which is not ionized. I think that we must be on the look-out for the unexpected reaction in this type of system, especially as so few molecules seem to be involved at one time, a point stressed by Krebs. We have found that aconitase is vastly more sensitive to fluorocitric acid when it is inside the mitochondria than in isolated solutions.

The sweep of modern biochemistry must astonish us all; witness the detailed knowledge of biosynthesis by enzymes now being obtained. Hopkins had always enormous belief in the potentialities of living matter, a belief justified by almost daily events. Bacteria hit back by becoming resistant to penicillin; flies by putting up with large concentrations of DDT. Even rabbits have learnt how to become immune against myxomatosis, and this has raised a big economic problem for Australia and even here, where rabbits used to do £50 million damage a year to crops. Knowledge of these biochemical mechanisms is wanted for practical reasons, but even more to complete the intellectual picture. My short session in an Institute of the Agricultural Research Council has focused for me the extraordinary biochemical adaptations in the ruminant, where in the rumen Pasteur's 'vie sans l'air' is in progress all the time and enables these animals to survive where we should perish.

Turning to our relations with other sciences, to the physiologist we must often still appear to pursue with our minces and particles a course so far removed from life that there is no hope of application. It must give them the kind of feeling that some of us have when we look at very modern art; I mean that kind of picture which depicts a mixture of playing cards and bits of limbs. Of course we can challenge this; in essence it is not different philosophically from the attempt to synthesize the physiological reactions of the whole animal from (say) a perfused-heart preparation.

But the point that I want to make particularly is that the behaviour of the intracellular enzyme must often be an active intruder in physiological experiments on the so called 'normal'. We are well aware of the importance of the cholinesterase type of enzyme in the functioning of nervous tissues; but in my view it is not only this type that matters, nor is our function as biochemists merely concerned with setting the right biochemical background for normality in the physiological experiment. I can focus my point best by asking you to listen to a rat heart poisoned with a long-chain fatty acid, fluorodecanoic acid, where the fluorocitric acid synthesized blocks the enzyme aconitase. You will hear that, as compared with the normal beat at 550/min., the heart is slowed and irregular. Whatever be the intermediate steps, the primary block here is in the enzyme aconitase, one of the enzymes of the tricarboxylic acid cycle, i.e. in the working of an intracellular enzyme; so that this explanation can be traced to a root in the Hopkins 1913 address. These matters are of course subtle and are mixed up with biophysics. But it is this type of experiment which convinces me that we must be on the look-out for the intrusion of intracellular biochemistry into some physiological experiments, and not only those concerned with the maintenance of stability of membrane potentials. What even is the final use of a membrane theory without knowledge of the constitution of the membrane which may itself be undergoing adjustments during life?

In pharmacology, pathology and medicine, it is certain that we have the clearest arguments for our importance. Biochemical change must precede pathological disturbance, and effects upon enzymes more often than not induce pharmacological action, whether this be by combination with active centres or by antimetabolite effects.

In conclusion, some general remarks seem necessary. There are still misunderstandings as to what biochemistry is about; fortunately, we can comfort ourselves with Emerson's saying that 'to be great is to be misunderstood'. In particular, objection has been taken to the term 'high energy bond', used in biochemistry to denote a molecule

with a valuable form of instability; the grounds of the objection are that the term does not mean this in chemistry. Surely someone could retort equally that the use of the term 'nucleophilic' in chemistry has always been most puzzling to the histologist, who has used nucleophilic stains since time immemorial.

There is some evidence also that the centuries'-old clash between vitalism and mechanism is taking on a new dress with a redefinition of vitalism—not as the entelechy of Driesch, but as a statement which appears to reduce to the idea that the state of organization in living matter is too complicated for expression in present chemical terms. It seems to be somewhat arbitrary to set a limit to the degree of complexity which physical and chemical knowledge may be held ultimately to embrace, and I doubt whether many chemists would do this. Perhaps in so far as living organisms become individuals, this will always set part of their activities outside statistical laws; certainly individuals or small groups count in the world's history. But surely our task is to continue to apply chemistry and physics to the inside of the cell until this fails us. Then, as Hopkins said, from time to time we may meet the spectre of vitalism. What biochemists have accomplished in the past is enough to show that any chemist entering the field with due humility will still accomplish much, and to justify the sustained faith and effort to put our feet on the right path. (I do not think that humility matters much, because Nature humbles us all.)

One thing is certain: now that we know a little more about the chemical nature of constituents of the cell, we are only just in a position to embark on the next stage, and it will take long even with present techniques to progress to our final goal. We are likely to remain inspired by the belief that if we put the mixture together properly the machine will go, if you like 'live'. Even if this happens, and it will be a long time before it could, we must be cautious in our philosophical interpretations and not go beyond our facts. Much of practical importance will emerge in our pilgrimage; but, best of all, the intellectual pursuit is worth while. *Labor ipse voluptas.* As Hopkins indicated in 1933, biochemistry is a better intellectual discipline than many of our friends in the humanities would think.

May I finish with some words from Joshua Reynolds, in which he refers to the pictorial arts: 'Study therefore the great works of the great masters, for ever.... Consider them as models which you are to imitate, and at the same time as rivals with whom you are to contend.'

I am indebted to Mrs B. E. Holmes for the photograph of her father, and to many others for their advice. The photo was enlarged from a group of three taken by a member of Harvard University, believed to be Dr Dill.

Studies on Sulphatases

23. THE ENZYMIC DESULPHATION OF TYROSINE *O*-SULPHATE*

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Suzuki, Takahashi & Egami (1957) have shown that the sulphated polysaccharide (charonin sulphate) of the mucous gland of *Charonia lampas* (*Triton nodiferus*) can be sulphated by a system comprising an arylsulphate, an arylsulphatase, the carbohydrate acceptor and at least one unknown factor. The physiological significance of this type of system is not yet clear, but if similar systems occur in other organisms it would seem reasonable to expect the presence in tissues of arylsulphates whose specific role would be that of sulphate donors. There are already indications (F. Egami, personal communication) of the presence of such an arylsulphate in the mucous gland of *Charonia*.

Although arylsulphates are normally present in appreciable amounts in mammalian urine, where they appear as end products of the metabolism of phenolic materials, they have not, with one notable exception, been shown to occur as normal constituents of mammalian tissues. The exception, tyrosine *O*-sulphate, has been shown by Bettelheim (1954) to be present in one of the two peptides which are released from bovine fibrinogen by the action of thrombin. Tyrosine *O*-sulphate is always present in appreciable amounts in normal urine (Tallan, Bella, Stein & Moore, 1955). It seemed to the present authors that tyrosine *O*-sulphate might conceivably act, *in vivo*, as a sulphate donor in a manner similar to that suggested by Suzuki *et al.* (1957), and it was therefore of interest to investigate the ability of the compound to act as a substrate for various arylsulphatase enzymes. A preliminary report of this work has already been made (Dodgson, Rose & Tudball, 1957).

MATERIALS AND METHODS

Substrates

The potassium salts of *p*-acetylphenyl sulphate (APS), *p*-nitrophenyl sulphate (NPS) and phenyl sulphate (PS) were prepared by the method of Burkhardt & Lapworth (1926). Dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) was prepared by the method of Roy (1953) as modified by Dodgson & Spencer (1956).

Tyrosine O-*sulphate*. The method described by Tallan *et al.* (1955) was modified slightly. Sulphuric acid (11 ml., sp.gr. 1·86) was stirred mechanically at −5° and L-tyrosine (2 g.) was added in small portions over a period of 20 min., great care being taken to avoid lumping. Stirring was then continued at −5° for a further period of 30 min. The reaction mixture was poured into 300 ml. of crushed ice and water containing 70 g. of $Ba(OH)_2$. Precipitated $BaSO_4$ was removed, and excess of Ba^{2+} ions were precipitated by addition of solid CO_2. After removal of $BaCO_3$ the solution was evaporated to dryness *in vacuo* at 40°, and the residue was dissolved in 60 ml. of water and passed through a column (8 cm. × 1 cm.) of Dowex 50 ion-exchange resin (Dow Chemical Co., Michigan, U.S.A.; 20–50 mesh, H^+ form). The eluate was adjusted to pH 5 with 5% KOH, concentrated to dryness *in vacuo* at 35°, dissolved in 2 ml. of water and treated alternately with small portions of ethanol and ether until 20 ml. of ethanol and 18 ml. of ether had been added. After 18 hr. at 0° the crystals of monopotassium tyrosine *O*-sulphate dihydrate were separated, and washed with 10 ml. of ethanol followed by 10 ml. of ether and dried *in vacuo*. Yield, 0·4 g. (Found: ester SO_4^{2-}, 28·1; K, 11·6. $C_9H_{10}O_6NSK,2H_2O$ requires ester SO_4^{2-}, 28·6; K, 11·6%). Ester SO_4^{2-} was determined as $BaSO_4$ after hydrolysis of the compound for 2 hr. at 100° with N-HCl. Potassium was determined by flame photometer. Loss in weight on drying at 110° *in vacuo* in the presence of P_2O_5 corresponded to $2H_2O$.

*Glycyl-*L*-tyrosine* O-*sulphate*. The method of preparation was essentially as described above except that 0·5 g. of glycyl-L-tyrosine (L. Light and Co.) was added over a period of 15 min. to 5 ml. of H_2SO_4 (sp.gr. 1·86) at −8° and stirring was continued for 25 min. The procedure described previously was then followed except that the residue was taken up in 30 ml. of water before passage through the Dowex column. Yield, 0·1 g. (Found: ester SO_4^{2-}, 23·3; K, 9·7. Monopotassium glycyl-L-tyrosine *O*-sulphate trihydrate (new compound), $C_{11}H_{13}O_7N_2SK,3H_2O$, requires ester SO_4^{2-}, 23·4; K, 9·5%). Loss in wt. at 110° corresponded to $3H_2O$.

Tyramine O-*sulphate*. Tyramine hydrochloride (L. Light and Co.) was dissolved in water and the free base precipitated by the addition of the requisite amount of K_2CO_3. The material was separated at the pump, washed with a little water and dried *in vacuo*.

The sulphation procedure was then exactly as described for glycyl-L-tyrosine *O*-sulphate except that, after passing through the Dowex column, the pH of the eluate was adjusted to 10·6 with 30% (w/v) KOH before concentrating. (Found: ester SO_4^{2-}, 32·4; K, 13·1. Monopotassium tyramine *O*-sulphate dihydrate (new compound), $C_8H_{10}O_4NSK,2H_2O$, requires ester SO_4^{2-}, 33·0; K, 13·1%). Loss in wt. at 110° corresponded to $2H_2O$.

* Part 22: Baum & Dodgson (1958).

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Paper chromatography. The homogeneity of preparations of tyrosine *O*-sulphate, glycyl-L-tyrosine *O*-sulphate and tyramine *O*-sulphate was checked by descending-paper chromatography on Whatman no. 1 paper, the solvent system being butan-1-ol–acetic acid–water in the proportions 125:30:125. After 16 hr. the chromatograms were dried and developed by spraying with a 0·25% solution of ninhydrin in acetone. R_F values of the esters and of the parent phenols are given in Table 1.

Ultraviolet-absorption spectra. It is known that sulphation with chlorosulphonic acid of compounds containing both phenolic hydroxyl and amino groups results in the sulphation of both groups. On the other hand, sulphation with H_2SO_4 at low temperature leaves the amino grouping unaffected although, under certain conditions, sulphonates may be formed. With the *O*-sulphates of tyrosine, glycyl-L-tyrosine and tyramine, it seems clear that the phenolic hydroxyl group only had been sulphated. Thus the analytical results reveal the presence of only one sulphate group per molecule and the compounds gave positive ninhydrin reactions indicating that the amino groupings were free. Desulphation was readily achieved under conditions (N-HCl at 100°) where sulphonates are stable and moreover all the compounds were readily desulphated by the arylsulphatases of Taka-diastase and *Alcaligenes metalcaligenes*.

The u.v.-absorption spectra (Fig. 1) also suggested that the compounds were true arylsulphates. It will be observed that each of the parent phenols possesses a characteristic absorption band in the region of 295 mμ, which can be attributed to the presence of the free phenolic grouping. The intensity of this band is greatly decreased in the corresponding sulphated compound indicating that sulphation of the phenolic group had occurred rather than ring sulphonation or sulphation of the amino group.

Table 1. *Chromatographic mobilities of* L-*tyrosine, glycyl-*L-*tyrosine and tyramine and their corresponding* O-*sulphate esters*

R_F values were obtained with butan-1-ol–acetic acid–water (125:30:125, by vol.). Experimental details are given in the text.

Compound	No. of experiments	Average R_F
Tyrosine	6	0·60
Tyrosine *O*-sulphate	6	0·12
Glycyl-L-tyrosine	4	0·45
Glycyl-L-tyrosine *O*-sulphate	4	0·25
Tyramine	4	0·67
Tyramine *O*-sulphate	4	0·30

Arylsulphatase preparations

A lactose-free preparation of Taka-diastase (Parke, Davis and Co. Ltd.) was used as a source of fungal arylsulphatase; the bacterial enzyme from *Alcaligenes metalcaligenes* was concentrated to give preparation B (Dodgson, Spencer & Williams, 1955) and limpet (*Patella vulgata*) arylsulphatase was concentrated to give powder D (Dodgson & Spencer, 1953*a*). Human arylsulphatases *A* and *B* were prepared from human liver according to the directions of Baum, Dodgson & Spencer (1958) and Dodgson & Wynn (1958) respectively.

Human-liver arylsulphatase C. The method of Dodgson, Spencer & Wynn (1956) was modified. Human liver (40 g.) obtained within 48 hr. after death was cut into small pieces and suspended in 150 ml. of water with the aid of a Townson and Mercer macerator. The suspension was centrifuged at 2 000 *g* (all values of *g* are average values) and 0° for 15 min. The cloudy supernatant was thrice alternately frozen and thawed before centrifuging for 30 min. at 54 450 *g* and 0° in the Spinco preparative ultracentrifuge (25 000 rev./min., head no. 30). The precipitate was washed (twice) by suspending in water and centrifuging, after which the precipitate was again suspended in water and subsequently freeze-dried.

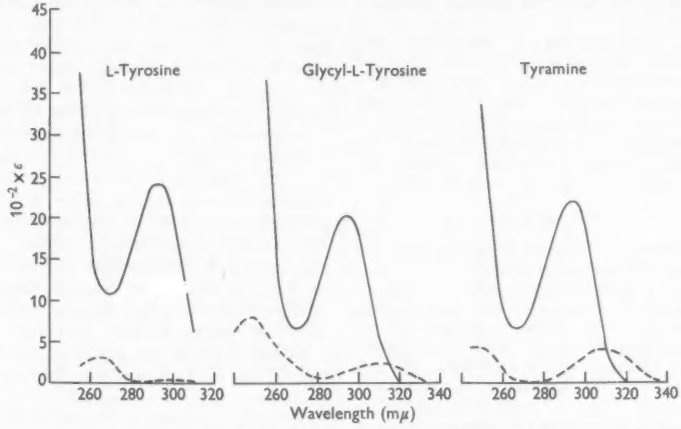

Fig. 1. Ultraviolet-absorption spectra of L-tyrosine, glycyl-L-tyrosine, tyramine and their corresponding *O*-sulphate esters. Spectra were determined in the presence of 0·1N-NaOH with the Hilger Uvispek instrument. Continuous lines represent the spectra of the parent phenols and broken lines the spectra of the *O*-sulphates.

Rat-liver arylsulphatase C. M.R.C. hooded rats (15) were killed by a blow on the head and the livers were removed as quickly as possible. The livers were then forced through a fine wire mesh in order to remove connective tissue. Portions of the resulting pulp (total wt. 160 g.) were each suspended in 20 ml. of isotonic sucrose solution with the aid of a glass homogenizer. The final suspension (1·5 l.) was centrifuged for 10 min. at 0° and 2000 *g* and the resultant cloudy supernatant was subsequently centrifuged for 30 min. at 0° and 7700 *g* on the Spinco preparative ultracentrifuge (8750 rev./min., head no. 21). Again a cloudy supernatant was obtained and this was clarified by centrifuging for 1 hr. at 0° and 54 450 *g* (25 000 rev./min., head no. 30). The red gelatinous sediment was separated and treated with 10 g. of Lissapol-N (Imperial Chemical Industries Ltd.) before adding sufficient ice-cold water to give a final volume of 250 ml. The pH of the mixture was adjusted to 9·8 with N-NaOH and the whole allowed to stand at 0° for 30 min. The pH was readjusted to 7·5 with acetic acid and the mixture was then treated with acetone at 0° until the concentration of acetone was 80% (v/v). After standing at 0° for 30 min. to allow the precipitate to flocculate the latter was separated by centrifuging at 0°, and washed with two 150 ml. portions of cold acetone and then dried *in vacuo*. The reddish brown material (1·5 g.) was suspended in 30 ml. of ice-cold water before centrifuging on the M.S.E. refrigerated centrifuge for 1 hr. at 0° and 20 000 *g*. The sediment was washed (twice) by suspending it in 30 ml. of ice-cold water and centrifuging as before. The final precipitate was then freeze-dried.

Ox-liver arylsulphatase C. This was prepared by a modification of the method of Roy (1956). Fresh ox liver (40 g.) was cut into small pieces and suspended in 600 ml. of ice-cold water with the aid of a Townson and Mercer macerator. The suspension was centrifuged for 5 min. at 0° and 2000 *g* and the resultant cloudy supernatant was then centrifuged on the Spinco ultracentrifuge for 30 min. at 0° and 20 000 *g* (13 750 rev./min., head no. 21). The sediment was washed (twice) by suspending it in 200 ml. of ice-cold water and centrifuging as before. The sediment was finally suspended in 200 ml. of ice-cold water containing 10 g. of Lissapol-N, the pH was adjusted to 9·0 with N-NaOH and the whole allowed to stand at 0° for 1 hr. The pH of the mixture was then adjusted to 7·5 with acetic acid before centrifuging for 90 min. at 0° and 54 450 *g* (25 000 rev./min., head no. 30). The supernatant was treated with acetone at 0° until the concentration of acetone was 80% (v/v) and the precipitated material was then collected, and washed and freeze-dried as described for the rat-liver preparation.

Determination of enzyme activity

Arylsulphatase activity towards NPS and NCS was measured by spectrophotometric estimation of the respective liberated phenols (Dodgson, Spencer & Thomas, 1955). Enzyme activity towards all other substrates was determined by estimating liberated sulphate by the method of Dodgson & Spencer (1953*b*). Except where otherwise stated, incubation of enzyme (0·6 ml.) and substrate (0·6 ml.) was for 1 hr. at 37·5° in the presence of 0·5 M-sodium acetate–acetic acid buffer adjusted to the appropriate pH. With each enzyme preparation preliminary experiments were made in order to establish the concentration of enzyme appropriate to the range of accuracy of the particular assay method used.

RESULTS

Table 2 records the optimum experimental conditions for the activity of the various enzymes towards tyrosine *O*-sulphate, PS, NPS and NCS. Michaelis constants (K_m) and maximum velocities ($V_{max.} = k_2[E]$) are also given and were determined by the method (case III) of Lineweaver & Burk (1934). Values of $V_{max.}$ for any one enzyme are relative values expressed in arbitrary units since, although the concentration of enzyme used was the same in each particular series of experiments, the absolute concentration $[E]$ was unknown.

Dodgson & Spencer (1957) have previously suggested that at least two types of arylsulphatases occur in nature. The type I arylsulphatases show a relatively high activity and affinity towards simple arylsulphates such as PS and NPS and are strongly inhibited by KCN. The type II enzymes, on the other hand, whilst appreciably active towards simple arylsulphates, show relatively greater affinity and activity towards more complex arylsulphates such as NCS. They are generally unaffected by KCN but are strongly inhibited by PO_4^{3-} and SO_4^{2-} ions. Table 2 shows that the type I arylsulphatases of Taka-diastase and of *Alcaligenes metalcaligenes* both exhibit appreciable activity and affinity towards tyrosine *O*-sulphate. With *Alcaligenes metalcaligenes* the activity of the enzyme towards tyrosine *O*-sulphate is about eight times as great as that towards PS, and the Taka-diastase enzyme is about half as active towards tyrosine *O*-sulphate as towards PS. On the other hand, the type II arylsulphatase of the limpet shows only feeble affinity and activity towards tyrosine *O*-sulphate relative to that shown towards the more complex substrate NCS. The type II arylsulphatases of human liver (arylsulphatases *A* and *B*) behave similarly, although with arylsulphatase *A* it was not possible to obtain figures of absolute quantitative significance owing to the anomalous kinetics exhibited by the enzyme (see Baum & Dodgson, 1958).

The finding that the type I arylsulphatases of *Alcaligenes* and Taka-diastase were appreciably active towards tyrosine *O*-sulphate was as expected. It was therefore particularly interesting to find that the type I arylsulphatases (arylsulphatase *C*) of human, rat or ox liver were apparently devoid of activity towards this substrate although all three enzymes were readily able to attack PS.

Although the length of the incubation period and the experimental conditions of pH, substrate concentration, type of buffer and buffer concentration were widely varied, no release of SO_4^{2-} ions from tyrosine *O*-sulphate could be detected. Moreover tyrosine *O*-sulphate when tested at various concentrations (0·005–0·02 M) failed to act as a competitive

Table 2. *Experimental conditions governing the activity of various arylsulphatases towards the potassium salts of 2-hydroxy-5-nitrophenyl-, p-nitrophenyl-, phenyl- and tyrosine O-sulphate*

NCS, 2-hydroxy-5-nitrophenyl sulphate; NPS, *p*-nitrophenyl sulphate; PS, phenyl sulphate; TOS, tyrosine *O*-sulphate.

Arylsulphatase	Buffer	Concn. of buffer (M)	Optimum pH	Substrate	Optimum concn. of substrate (mM)	$10^4 K_m$	Relative $V_{max.}$	Incubation period (hr.)
Type I enzymes								
Alcaligenes metalcaligenes	Tris–acetic acid	0·2	8·0	NCS	15·0	2·2	1·2	1·0
			8·75	NPS	1·5	2·3	16·5	1·0
			8·75	PS	3·5	6·1	1·0	1·0
			9·0	TOS	10·0	3·2	7·6	1·0
Taka-diastase	Acetate	0·5	5·8	NCS	20·0	7·3	2·0	1·0
			6·3	NPS	15·0	3·7	2·0	1·0
			6·2	PS	20·0	23·0	1·0	1·0
			6·3	TOS	15·0	56·2	0·5	1·0
Rat-liver *C**	Acetate	0·5	7·4	NPS	2·5	7·0	4·0	6·0
			7·0	PS	40·0	46·0	1·0	6·0
			—	TOS	—	No activity		6·0
Ox-liver *C**	Tris–acetic acid	0·2	8·0	NPS	8·0	20·0	18·0	2·0
			8·0	PS	20·0	95·2	1·0	2·0
			—	TOS	—	No activity		16·0
Human-liver *C**	Tris–acetic acid	0·2	7·3	NPS	8·0	28·0	6·8	5·0
			7·5	PS	100·0	450·0	1·0	5·0
			—	TOS	—	No activity		16·0
Type II enzymes								
Limpet (*Patella vulgata*)	Acetate	0·5	5·5	NCS	5·0	6·9	4·5	1·0
			5·5	NPS	55·0	60·0	1·5	1·0
			5·7	PS	60·0	125·0	1·0	1·0
			5·5	TOS	60·0	180·0	0·5	1·0
Human-liver *B*	Acetate	0·5	6·0	NCS	10·0	12·3	15·0	1·0
			6·0	NPS	>50·0	124·0	1·0	3·0
			—	PS	—	No activity		16·0
			—	TOS	—	No activity		16·0
Human-liver *A*†	Acetate	0·5	5·0	NCS	5·0	—	15·7	1·0
			5·3	NPS	40·0	—	9·3	1·0
			5·3	PS	40·0	—	1·0	1·0
			5·3	TOS	40·0	—	2·6	1·0

* No figures are available for NCS. The activity shown towards NCS by these enzyme preparations can be almost completely inhibited by PO_4^{3-} ions and is therefore probably due to the presence of small amounts of arylsulphatases *A* and *B* (see Dodgson, Rose & Spencer, 1957).

† Owing to the anomalous kinetics of this enzyme it was not possible to determine Michaelis constants and the relative $V_{max.}$ figures quoted actually represent μg. of SO_4^{2-} ions liberated in 1 hr. by a fixed concentration of enzyme.

inhibitor of the hydrolysis of NPS by rat-liver arylsulphatase *C*. On the contrary, slight enzyme increases (up to 10%) in activity were observed under these circumstances. Similar results were obtained when APS was substituted for NPS, although the activatory effect of tyrosine *O*-sulphate was less pronounced then.

Experiments with soluble preparations of arylsulphatase C. Mammalian-liver arylsulphatase *C*, irrespective of the species of origin, is a microsomal enzyme which cannot be solubilized by the usual mild procedures. It therefore seemed possible that the failure of the enzyme to hydrolyse tyrosine *O*-sulphate might be related to the insoluble particulate nature of the enzyme. Accordingly, rat-liver arylsulphatase *C* was solubilized by treatment with detergents as described by Dodgson, Rose, Spencer & Thomas (1957). Three different detergents were used, the non-ionic Lissapol-N, the cationic cetylpyridinium bromide (L. Light and Co.) and the anionic Teepol 530 (Shell Chemicals Ltd.). It was not possible to test directly the activity of the solubilized enzyme against tyrosine *O*-sulphate since in each case the detergent interfered markedly with recoveries of SO_4^{2-} ions. It was therefore necessary to test the ability of tyrosine *O*-sulphate to act as a competitive inhibitor of the hydrolysis of NPS by the solubilized enzyme. These experiments were performed at pH 7·4 in the presence of a 0·0025M-solution of NPS in 0·5M-sodium acetate–acetic acid mixture (see Table 2), the concentration of tyrosine *O*-sulphate being varied between 0·005 and 0·02M. Irrespective of the length of incubation period used tyrosine *O*-sulphate did not inhibit the hydrolysis of NPS by any of the enzyme preparations. Indeed, with the Lissapol-N- and Teepol 530-treated enzyme preparations, increases of up to 10% of enzyme activity towards NPS was observed in the presence of tyrosine *O*-sulphate. It seems clear that the failure of arylsulphatase *C* to hydrolyse tyrosine *O*-sulphate is not related to the insoluble nature of the enzyme.

*Experiments with glycyl-*L*-tyrosine* O-*sulphate and tyramine* O-*sulphate*. Dodgson, Spencer & Williams (1956) have shown for the arylsulphatase of *Alcaligenes metalcaligenes* that the activity of this enzyme is markedly affected by the nature of any substituent groups present in the aromatic nucleus of the arylsulphate substrate. It was therefore of interest to see whether tyrosine *O*-sulphate could be induced to act as a substrate for arylsulphatase *C* by slightly modifying the side chain of the compound, and attempts were made to prepare various sulphate esters whose structures were closely related to that of tyrosine *O*-sulphate. The majority of these attempts were unsuccessful for a variety of reasons. Thus the ethyl and methyl esters of tyrosine could not be sulphated without loss of ethyl or methyl groups, the hydroxamic acid derivative could not be sulphated and it proved impossible to prepare the *N*-acetyl derivative of tyrosine *O*-sulphate in a form sufficiently pure for enzyme experiments. Attempts to prepare the 3-nitro- and the 3:5-di-iodo-derivatives of tyrosine *O*-sulphate were also unsuccessful. It did prove possible, however, to prepare glycyl-L-tyrosine *O*-sulphate (where the α-amino grouping of tyrosine is participating in a peptide linkage) and tyramine *O*-sulphate (which contains no carboxyl group). Glycyl-L-tyrosine *O*-sulphate was readily desulphated by *Alcaligenes metalcaligenes* or Taka-diastase at a similar rate to tyrosine *O*-sulphate. However, no hydrolysis of the compound by rat, ox or human arylsulphatase *C* could be achieved under a wide variety of experimental conditions. Tyramine *O*-sulphate was readily hydrolysed by the arylsulphatase of Taka-diastase at about twice the rate at which tyrosine *O*-sulphate was desulphated. It was also hydrolysed by rat-, ox- or human-liver arylsulphatase *C* but at an extremely slow rate. In the most favourable experiment a preparation of rat-liver arylsulphatase *C* liberated only 11·4 μg. of SO_4^{2-} ions from 0·01M-tyramine *O*-sulphate in the presence of 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer, pH 6·2, in a total incubation time of 22 hr. at 37·5°. The same concentration of enzyme liberated 457 μg. of SO_4^{2-} ions from NPS under optimum conditions in 1 hr. at 37·5°.

DISCUSSION

The occurrence of tyrosine *O*-sulphate in one of the two peptides which are released from bovine fibrinogen by the action of thrombin seems to have been established with reasonable certainty (Bettelheim, 1954), although the physiological significance of this finding is quite obscure. Tyrosine *O*-sulphate thus occupies a unique position among the naturally occurring arylsulphates, which otherwise appear to arise solely as end products of the metabolism of phenols. In another respect tyrosine *O*-sulphate appears to be a unique arylsulphate since it is reported (Segal, 1957 and personal communication) that tyrosine itself cannot be sulphated by the adenosine 3′-phosphate 5′-sulphatophosphate (3′-phosphoadenosine 5′-phosphosulphate; PAPS)–phenolsulphokinase system which is responsible for the synthesis of other arylsulphates. Workers in the Cardiff Laboratories have also attempted, without success, to sulphate tyrosine via the PAPS–phenolsulphokinase route. Now in a third respect tyrosine *O*-sulphate appears to be unique since it is completely unaffected by the mammalian arylsulphatase (arylsulphatase *C*) which might, from theoretical considerations, have been expected to hydrolyse it. Indeed, of the three known arylsul-

phatases present in mammalian liver only arylsulphatase *A* is active (feebly) towards tyrosine *O*-sulphate. It is clear that in mammals tyrosine *O*-sulphate, as such, is unlikely to be capable of acting as a sulphate donor in a system similar to that described for *Charonia lampas* by Suzuki *et al.* (1957). It is possible, of course, that sulphation of tyrosine can occur only via the PAPS–phenolsulphokinase system when both the α-amino and carboxyl groups of the tyrosine molecule are participating in peptide linkages. In a similar way it may be that blockage of both α-amino and carboxyl groups is a necessary prerequisite for the desulphation of tyrosine *O*-sulphate by mammalian arylsulphatase *C*. Attempts have been made to investigate this possibility with a sulphated specimen of β-lactoglobulin (containing about 2% of tyrosine-bound ester SO_4^{2-} ions) but the results obtained so far have been inconclusive.

Other arylsulphatases are able to desulphate tyrosine *O*-sulphate readily, the arylsulphatase of *Alcaligenes metalcaligenes* being particularly active in this respect. The activity of this enzyme towards arylsulphate esters is increased by the presence of an electrophilic substituent group (e.g. $-NO_2$) in the aromatic nucleus, as a result of the withdrawal of electrons from the environment of the ester SO_4^{2-} ion group (Dodgson, Spencer & Williams, 1956). On the other hand nucleophilic substituent groups (e.g. $-NH_2$) have the reverse effect. The fact that tyrosine *O*-sulphate is hydrolysed by the *Alcaligenes* enzyme at a rate approximately eight times as great as PS suggests that at the pH of the experiment (9·0) the strongly electrophilic carboxyl group of tyrosine *O*-sulphate, even though insulated from the aromatic ring by a two-carbon chain, is still able in some way to influence the distribution of electrons in the ester SO_4^{2-} ion grouping.

SUMMARY

1. A study has been made of the ability of tyrosine *O*-sulphate to act as a substrate for various arylsulphatase enzymes.

2. Tyrosine *O*-sulphate is not a substrate for two of the mammalian arylsulphatases (arylsulphatases *B* and *C*) and is only feebly attacked by the third mammalian enzyme, arylsulphatase *A*.

3. The failure of mammalian arylsulphatase *C* to hydrolyse tyrosine *O*-sulphate is unexpected and not in accord with theoretical predictions. The enzyme is also inactive towards glycyl-L-tyrosine *O*-sulphate and only feebly active towards tyramine *O*-sulphate.

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The Fermentation of Lactic Acid by a Gram-negative Coccus

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A number of types of bacteria convert lactate into propionate anaerobically and the mechanism of this reaction has been widely studied. Johns (1951*a*), using washed cells of *Veillonella gazogenes* (*Micrococcus lactilyticus*), found that lactate was fermented only in the presence of carbon dioxide and that the amount of propionate formed increased with the carbon dioxide tension in the medium. When lactate was fermented in the presence of $^{13}CO_2$, [1-^{13}C]propionate was produced. Fumarate and L-malate were both attacked by *V. gazogenes*, giving a mixture of propionate and acetate; succinate was quantitatively decarboxylated to propionate and carbon dioxide. Johns concluded that in this organism the synthesis of propionate involves the formation of a dicarboxylic acid with four carbon atoms by a carbon dioxide-fixation reaction, and that this acid is reduced to succinate which is then decarboxylated to propionate. Whiteley (1953*a*–*c*) studied the decarboxylation reaction in more detail, using cell-free extracts of *V. gazogenes*. She found that before decarboxylation succinate is converted into succinyl-coenzyme A. The succinyl-coenzyme A is then decarboxylated, yielding carbon dioxide and propionyl-coenzyme A. Transfer of coenzyme A from the latter to succinate yields propionate and succinyl-coenzyme A, thus enabling the reaction to proceed.

A similar mechanism may operate in *Propionibacterium* sp. (Delwiche, 1948; Delwiche, Phares & Carson, 1953, 1954; Johns, 1951*b*; Leaver, Wood & Stjernholm, 1955), but evidence has been presented (Wood, Stjernholm & Leaver, 1955) that it may not be the sole pathway of propionate formation in these bacteria.

By contrast, *Clostridium propionicum* appears to form propionate by a more direct mechanism without production of intermediary dicarboxylic acids. This organism differs from both *V. gazogenes* and *Propionibacterium* sp. in so far as it ferments acrylate. Johns (1952) showed that washed cells of *C. propionicum* grown on lactate neither decarboxylated succinate nor fermented DL-malate and fumarate; further, the fermentation of lactate was not influenced by the tension of carbon dioxide and the propionate formed from lactate in the presence of $^{13}CO_2$ was unlabelled.

Leaver *et al.* (1955) showed that *C. propionicum* fermented [3-^{14}C]lactate to [3-^{14}C]propionate and [2-^{14}C]acetate, indicating a direct reduction and oxidation of the lactate. Succinate behaves as a symmetrical molecule in biological systems, and thus if it is an intermediate in the formation of propionate an equimolar mixture of [3-^{14}C]- and [2-^{14}C]-propionate would have been produced.

The rumen organism LC (Elsden, Volcani, Gilchrist & Lewis, 1956) ferments DL-lactate with the formation of hydrogen, carbon dioxide, acetate, propionate, *n*-butyrate, *n*-valerate and small amounts of *n*-caproate; it also ferments acrylate (Elsden & Lewis, 1953). Elsden *et al.* (1956) found that neither washed-cell suspensions nor growing cultures decarboxylated succinate and they suggested that lactate is converted into propionate by the direct route with acrylate or a derivative of acrylate as an intermediate.

The fermentation of [2-^{14}C]lactate by washed-cell suspensions and cell-free extracts of LC has now been investigated and the results given below support the view that lactate is converted directly into propionate.

MATERIALS AND METHODS

Growth media and preparation of washed suspensions. The strain LC1 was used in all experiments and was maintained on the following medium: 2% (v/v) of 70% DL-sodium lactate, 0·4% (w/v) of Difco-yeast extract, 0·03% (w/v) of thioglycollic acid and 0·1% (w/v) of Davis New Zealand agar (J. W. Towers and Co. Ltd., Manchester) in tap water. The pH was adjusted to 7·0 before autoclaving.

For experiments with washed cells an overnight tube culture of LC in the above medium was inoculated into 500 ml. of the following liquid medium: 2% (v/v) of 70% DL-sodium lactate, 0·4% (w/v) of Difco-yeast extract and 0·02% of sodium dithionite in tap water, The dithionite was sterilized separately by Seitz filtration and added to the rest of the medium immediately before inoculation. The inoculated medium was incubated for 20–24 hr. at 37° under H_2. The cells were harvested and washed by the procedure described by Elsden & Lewis (1953).

Preparation of cell-free extracts. Two types of extract were used, one prepared from freshly harvested washed cells, the other from dried cells. A paste of washed cells was crushed in the Hughes press previously cooled to approx. −20° (Hughes, 1951), and the debris thoroughly mixed with freshly boiled 0·1M-phosphate buffer (a KH_2PO_4–

K_2HPO_4 mixture was used throughout), pH 6·5, and containing in addition 0·02% (w/v) of $Na_2S,9H_2O$; 1 ml. of the buffer mixture was used/g. wet wt. of cells. The suspension was centrifuged at 2° in the high-speed head of an International refrigerated centrifuge for 30 min. at 14 000 *g* and the supernatant, which contained the activity, was stored under air or N_2 at −20°. These extracts contained approx. 50 mg. of protein/ml., as estimated by the method of Stadtman, Novelli & Lipmann (1951).

Dried cells, prepared as described by Walker (1958), were extracted as follows: a 10% (w/v) suspension of dried cells in water was incubated under H_2 for 1 hr. at 37°. The suspension was then centrifuged at 14 000 *g* for 30 min. and the supernatant, which contained the activity, was stored under air or N_2 at −20°. These extracts contained approx. 20 mg. of protein/ml.

Fermentation procedure. Unless otherwise stated, the fermentations were carried out in 100 ml. flasks similar to those used by Lewis & Elsden (1955), except that one of the side arms was fitted with a stopper carrying a stop-cock. The suspension was placed in the main compartment and the substrate in one of the side arms; the other side arm contained 0·7 ml. of 10N-H_2SO_4. The flasks were gassed with O_2-free N_2 and were then shaken in a water bath at 37°. After equilibration, the pressure within the flasks was released by momentarily opening the main cock. The substrate was then tipped from the side arm. At the end of the experiment the contents were acidified by mixing with the H_2SO_4.

Assay of radioactive carbon dioxide. A CO_2-absorption tube (Sakami, 1955) containing 2N-NaOH soln. (CO_2-free) was fitted to the reaction vessel and the CO_2 produced swept out in a stream of CO_2-free air. The total CO_2 formed was measured manometrically as follows: duplicate samples (1 ml.) from the absorber were pipetted into Warburg manometer cups and the CO_2 was liberated by acidification with 0·5 ml. of 5N-H_2SO_4. Further samples of NaOH soln. were taken from the absorber, and diluted with water, and carrier Na_2CO_3 was added and barium carbonate plates were prepared and assayed for radioactivity as described by Ormerod (1956). The radioactivity was measured with a Geiger–Müller end-window counter. The activity was corrected for background and self-absorption and the results were expressed as specific activities (counts/min./μmole) at infinite thinness.

Volatile fatty acids. After removal of the CO_2 from the reaction vessel the contents were centrifuged and samples of the supernatant were steam-distilled in a Markham apparatus. The distillate was aerated with CO_2-free air for 2–3 min. and the total acids were determined by titration with standard NaOH soln. (approx. 0·01N). The neutralized solution was evaporated to dryness in a boiling-water bath and the residue dissolved with scratching in 2–3 ml. of an acidified 5% (v/v) solution of butanol in chloroform (2 drops of conc. H_2SO_4 in 50 ml. of solution). The individual fatty acids were separated on Celite columns buffered at either pH 6·5 or pH 7·6, according to Bueding & Yale (1951). Fractions (5 ml.) were collected and titrated with standard NaOH soln. (approx. 4 mN) in a stream of CO_2-free air, with phenol red as indicator. The fractions containing the individual neutralized acids were pooled and evaporated to a final volume, usually 10 ml., and samples taken for assay of the radioactivity.

Where the total radioactivity of the fatty acid was required, a sample to which sufficient carrier acid had been added to bring the total carbon content to 0·5 mg.-atom was dried in a combustion flask and the residue oxidized to CO_2 (Ormerod, 1956). The CO_2 produced was assayed for radioactivity as described above. Since the combustion procedure is quantitative, the amount of CO_2 produced was not measured and specific activities were calculated from the μmoles of acid oxidized.

Fatty acids were degraded by the method of Phares (1951) as modified by Sakami (1955). This procedure is based on the Schmidt reaction. In this reaction, the carboxyl of the fatty acid is converted into CO_2 and the remainder of the carbon chain is converted into an amine which, on oxidation with permanganate, is converted into a fatty acid containing one less carbon atom.

To a sample of fatty acid contained in a reaction tube, sufficient carrier acid was added to bring the total to 0·5 m-mole. The mixture was then taken to dryness and solid sodium azide, activated according to Sakami (1955), was added; the vessel was thoroughly cooled in ice and the H_2SO_4 added. Sakami recommends the gradual addition of the azide after the addition of the acid, but this was found inconvenient and the recoveries of CO_2 by our method were similar to those given by Sakami. The amine resulting from the final decarboxylation was recovered by distillation and oxidized as the sulphate.

Unlike results with the method for complete combustion, there were significant losses at each step in the degradation (up to 25% during the conversion of the amine into the carboxylic acid). The amount of CO_2 produced by decarboxylation and by combustion of the final amine was measured by direct weighing. If x counts/min. is the radioactivity of the sample counted (corrected for background and self-absorption) then the radioactivity of the starting material was: x (moles of acid taken after addition of carrier acid/moles of CO_2 produced by decarboxylation or combustion). When the amount of fatty acid in the sample before addition of carrier is known, the specific activity may be calculated.

Pyruvate. Where the specific activity of pyruvate was required the following procedure was adopted. At the end of the incubation period, the reaction mixture was acidified. Samples were deproteinized with trichloroacetic acid (final concn. 5%, w/v), centrifuged and 10 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl was added to the supernatant. After 24 hr. at 2°, the precipitated pyruvic acid 2:4-dinitrophenylhydrazone was filtered off, washed with 2N-HCl and dissolved in 20 ml. of ethyl acetate. The acid 2:4-dinitrophenylhydrazone was extracted into 30 ml. of 10% (w/v) Na_2CO_3 soln., from which it was precipitated by the dropwise addition of conc. H_2SO_4. After it had stood at 2° for 24 hr. the precipitate was filtered off, dissolved in ethyl acetate and transferred to a tared container. The ethyl acetate was evaporated and the 2:4-dinitrophenylhydrazone dried *in vacuo* and weighed. To determine its radioactivity the derivative was dissolved in a measured volume of ethyl acetate and samples, after drying, were oxidized in the usual manner.

Where the specific activity of the pyruvate carboxyl group was required, samples of the acidified reaction mixture, adjusted to pH 5·0, were placed in Warburg cups with two side bulbs, in one of which was placed 0·5 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl and in the other 0·2 ml. of yeast carboxylase.

Table 1. *Fermentation of* DL-[2-^{14}C]*lactate by washed cells of LC*

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (150 mg. dry wt. of cell material) in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% of $Na_2S,9H_2O$; 175·5 μmoles of DL-[2-^{14}C]lactate [specific activity (S.A.) 4190 counts/min./μmole] in 1·25 ml. of water, or 1·25 ml. of water in the control, added from one side bulb after equilibration. Gas phase N_2; temp. 37°. Reaction time 0·75 hr.

Product	Amount produced (μmoles)		Specific activity (counts/min./μmole)		S.A. of product (corr.) / S.A. of lactate
	Uncorr.	Corr.	Uncorr.	Corr.	
Carbon dioxide	170	120	6·8	9·6	0·0
Caproate	6·5	6·5	8800	8800	2·1
Valerate	52·5	42·1	6750	8390	2·0
Butyrate	31·1	24·7	5990	7540	1·8
Propionate	11·8	5·9	2700	5420	1·3
Acetate	21·0	6·9	1380	4180	1·0

Carbon recovered 95·5%; activity recovered 94·5%.

After equilibration for 10 min., the carboxylase was added and when gas evolution had ceased the pressure was noted and the 2:4-dinitrophenylhydrazine tipped into the main compartment to fix the aldehyde produced. The CO_2 was then transferred to an absorber in a stream of CO_2-free air, converted into barium carbonate and counted as described above.

Substrates. Sodium DL-lactate was prepared by neutralizing a boiling solution of A.R. lactic acid with dilute NaOH soln. and standardized by the method of Elsden & Gibson (1954). Sodium acrylate was prepared by steam-distilling an acidified solution of 60% acrylic acid (Light and Co., Colnbrook, Bucks) and titrating with standard alkali in a stream of CO_2-free air. The stock solution was standardized by hydrogenation with colloidal palladium (Harrison, 1939). Crystalline sodium pyruvate was prepared from redistilled pyruvic acid (Light and Co.) and standardized manometrically with yeast carboxylase (Westerkamp, 1933). The sodium salts of the fatty acids were obtained by neutralizing solutions of the redistilled acids. Sodium [^{14}C]carbonate and sodium DL[2-^{14}C]lactate were obtained from The Radiochemical Centre, Amersham, Bucks. A radioautograph, prepared from a chromatogram of the radioactive lactate run in the solvent butanol–acetic acid–water (4:1:5, by vol.), gave one spot with an R_F value (0·72) identical with that of A.R. lactic acid.

RESULTS

Fermentation of DL-[2-^{14}C]*lactate.* The yields and specific activity of the products of DL-[2-^{14}C]lactate fermentation by washed cells of LC are shown in Table 1. The relative proportions of the fatty acids formed varied markedly with different batches of cells. In this particular experiment the suspension had a high synthetic activity and the major proportion of the total fatty acids formed consisted of valeric and butyric acids. The CO_2 and fatty acids produced, corrected for endogenous formation, accounted for 95·5% of the carbon added as lactate.

The CO_2 was unlabelled. The corrected specific activities of acetate and propionate were each approximately equal to the specific activity of the added lactate and the corrected specific activities of caproate, valerate and butyrate were each approximately twice that of the lactate. The results suggest that both acetate and propionate were produced directly from lactate and that caproate, valerate and butyrate were each synthesized from two molecules of lactate. Evidence that this was not so for caproate will be presented later (Table 3).

Table 2. *Distribution of* ^{14}C *in acetate and propionate produced from the fermentation of* DL-[2-^{14}C]*lactate by washed cells of LC*

Experimental conditions were the same as described for Table 1.

Compound or group	Specific activity (counts/min./μmole)	Percentage of total activity
Acetate	4180	1000
$-CO_2H$	3885	93·0
CH_3-	27	0·7
Propionate	5420	100
$-CO_2H$	121	2·2
$-CH_2-$	4680	86·5
CH_3-	44	0·8

Table 3. *Distribution of* ^{14}C *in butyrate, valerate and caproate produced from the fermentation of* DL-[2-^{14}C]*lactate by washed cells of LC*

Experimental conditions were the same as described for Table 1.

Compound or group	Specific activity (counts/min./μmole)	Percentage of total activity
Butyrate	7540	100
$-CO_2H$	3870	51·4
$CH_3\cdot[CH_2]_2-$	4270	56·6
Valerate	8390	100
$-CO_2H$	3760	44·7
$CH_3\cdot[CH_2]_3-$	3880	46·3
Caproate	8800	100
$-CO_2H$	2880	32·8
$CH_3\cdot[CH_2]_4-$	5350	60·9

Table 4. *Fermentation of* DL-[2-^{14}C]*lactate by cell-free extracts of LC*

Experiments were carried out in manometer cups containing 1·6 ml. of cell-free extract (13·6 mg. of bacterial protein) in 0·1 M-phosphate buffer, pH 6·5, containing 0·01% of $Na_2S,9H_2O$; 44·2 μmoles of DL-[2-^{14}C]lactate [specific activity (S.A.) 6650 counts/min./μmole] in 0·6 ml. of water, or 0·6 ml. of water in the control, were added from the side bulb. Gas phase N_2; temp. 37°. Reaction time 2·5 hr. Reaction was stopped by addition of 0·5 ml. of 5 N-H_2SO_4. Bacterial extract was prepared from crushed cells.

Product	Amount produced (μmoles) Uncorr.	Corr.	Specific activity (counts/min./μmole) Uncorr.	Corr.	S.A. of product (corr.) / S.A. of lactate	Percentage of total activity
Acetate	19·7	17·5	5340	6010	0·91	100
$-CO_2H$	—	—	5160	5800	—	96·6
CH_3-	—	—	38	43	—	0·7
Propionate	25·6	24·7	5890	6100	0·92	100
$-CO_2H$	—	—	30	31	—	0·5
$-CH_2-$	—	—	5430	5630	—	92·4
CH_3-	—	—	57	59	—	1·0

The distribution of radioactivity in the acetate and propionate molecules is shown in Table 2. The acetate was labelled exclusively in the carboxyl group. Propionate was labelled almost entirely in the methylene group. There are two possible explanations for the very slight activity of the propionate carboxyl group: (*a*) during the degradation there may have been a very small carry-over of methylene carbon in the initial decarboxylation; (*b*) the propionate, separated by the chromatography procedure, may have contained traces of acetate, the carboxyl group of which would degrade with the carboxyl group of propionate. The methyl group of propionate was not significantly labelled.

The specific activities of the carboxyl group and the remainder of the molecule were determined for butyrate, valerate and caproate and compared with the specific activity of the complete molecule respectively (Table 3). The specific activity of butyrate was twice that of the butyrate carboxyl; the specific activity of valerate was twice that of the valerate carboxyl and the specific activity of caproate was three times that of the caproate carboxyl.

The fermentation of DL-[2-^{14}C]lactate was repeated with cell-free extracts of LC (Table 4). Cell-free extracts ferment lactate to CO_2, H_2, propionate and acetate. In this experiment, only the acetate and propionate formed were isolated and estimated; the activity of the CO_2 evolved was not measured. The results confirm those obtained with whole cells. Again the acetate was labelled exclusively in the carboxyl group and the propionate labelled exclusively in the methylene group. The specific activity of the acetate and propionate were each approximately equal to the specific activity of the added lactate.

The mechanism of valerate synthesis was studied more closely. Elsden & Lewis (1953) observed that washed-cell suspensions of LC synthesized appreciable amounts of valerate when pyruvate was fermented under H_2 in the presence of added propionate. Valerate, isolated from the fermentation products of pyruvate plus [1-^{14}C]propionate, was decarboxylated and the specific activities of both the carboxyl group and the butylamine resulting from the decarboxylation were compared with the specific activity of the total molecule (Table 5). The carboxyl group was unlabelled.

Table 5. *Distribution of* ^{14}C *in valerate produced from the fermentation of pyruvate plus* [1-^{14}C]*propionate by washed cells of LC*

Experiments were carried out in 100 ml. fermentation flasks containing 3 ml. of bacterial suspension (71 mg. dry wt. of cell material) in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% of $Na_2S,9H_2O$; 150 μmoles of pyruvate plus 110 μmoles of [1-^{14}C]propionate (specific activity 550 counts/min./μmole) in 2·1 ml. of water were added from one side bulb after equilibration. Gas phase H_2; temp. 37°. Reaction time 1·5 hr.

Compound or group	Specific activity (counts/min./μmole)	Percentage of total activity
Valerate	466	100
$-CO_2H$	15	3·5
$CH_3\cdot[CH_2]_3-$	456	98·0

To test whether CO_2 played a part in the formation of propionate (cf. fermentation by *V. gazogenes*) lactate was fermented in the presence of $^{14}CO_2$ and the amount of the isotope incorporated into the fatty acids measured. The results are shown in Table 6. It will be seen that, whereas the acetate and butyrate contained practically no ^{14}C, the specific activities of both propionate and valerate were approximately 6% of that of the final CO_2. All the activity of the propionate was found in the carboxyl group.

The experiment was repeated with a different batch of cells and with acrylate as substrate. Determination of the specific activities of propionate and acetate showed no significant incorporation of activity into either acid (Table 7).

To account for the slight labelling of the propionate formed from lactate fermented in the presence of $^{14}CO_2$, extracts of LC were tested for their ability to catalyse an exchange reaction between pyruvate and $^{14}CO_2$ (Table 8). Such extracts break down pyruvate quantitatively to CO_2, H_2 and acetate. Although the pyruvate acquired some of the ^{14}C label, equilibration with the $^{14}CO_2$ was incomplete even after incubation for 60 min., when one-half of the added pyruvate had been decomposed. All the activity of the pyruvate resided in the carboxyl group. A control with boiled extract showed no exchange between pyruvate and CO_2.

DISCUSSION

Two mechanisms have been described for the formation of propionate from lactate and, if [2-^{14}C]lactate is the substrate, then the distribution of the isotope in the propionate formed by these two mechanisms will be that shown in Fig. 1. The experiments described above show that in LC the conversion of lactate into propionate proceeds by mechanism *A*. The evidence for this contention is that fermentation of DL-[2-^{14}C]lactate both by whole cells and by cell-free extracts yields [2-^{14}C]propionate, as predicted by mechanism *A*. Had the propionate been formed via succinate as in *V. gazogenes* and *Propionibacterium* sp. then [2-^{14}C]propionate and [3-^{14}C]propionate would have been formed and this mixture would degrade as $^{14}CH_3 \cdot {}^{14}CH_2 \cdot CO_2H$, as was found by Leaver *et al.* (1955).

Table 6. *Incorporation of $^{14}CO_2$ into products of lactate fermentation by washed cells of LC*

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (50 mg. dry wt. of cell material) in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% of $Na_2S,9H_2O$; 194 μmoles of DL-lactate plus 32 μmoles of [^{14}C]-carbonate in 1·05 ml. of water, or 32 μmoles of [^{14}C]-carbonate in 1·05 ml. of water in the control, were added from one side bulb after equilibration. Gas phase N_2; temp. 37°. Reaction time 1·5 hr.

Product	Amount produced (μmoles)	Specific activity (counts/min./μmole)	Equilibration (%)
Carbon dioxide	186	37 300	—
Valerate	26·4	2 120	5·7
Butyrate	20·3	169	0·5
Propionate	51·8	2 340	6·3
$-CO_2H$	—	2 510	—
$CH_2 \cdot CH_2-$	—	35	—
Acetate	45·0	112	0·3

Table 7. *Investigation of incorporation of $^{14}CO_2$ into products of acrylate fermentation by washed cells of LC*

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (62·5 mg. dry wt. of cell material) in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% of $Na_2S,9H_2O$; 148 μmoles of acrylate plus 32 μmoles of [^{14}C]carbonate in 1·05 ml. of water, or 32 μmoles of [^{14}C]carbonate in 1·05 ml. of water in the control, were added from one side bulb after equilibration. Gas phase N_2; temp. 37°. Reaction time 1 hr.

Product	Amount produced (μmoles)	Specific activity (counts/min./μmole)	Equilibration (%)
Carbon dioxide	125	60 500	—
Propionate	86·1	71	0·11
Acetate	40·7	44	0·07

Table 8. *Incorporation of $^{14}CO_2$ into pyruvate during its fermentation by cell-free extracts of LC*

Manometer cups with double side bulbs contained 0·3 ml. of cell-free extract (6·6 mg. of bacterial protein) in water, 0·5 ml. of 0·1 M-phosphate buffer, pH 6·5, and 0·3 ml. of water. One side bulb, fitted with a turnover-type rubber stopper (Ormerod, 1956), contained 0·2 ml. of 0·5 M-[^{14}C]carbonate; the other side bulb contained 0·5 ml. of 0·4 M-pyruvate. Gas phase N_2; temp. 37°. After equilibration, 0·2 ml. of 5 N-H_2SO_4 was injected into the side bulb containing carbonate. Pyruvate was tipped into the main compartment after equilibration for a further 15 min. After incubation for 15, 30 and 60 min. periods, acid was tipped from the side arm. The control contained boiled extract and was incubated for 60 min. The extract was prepared from dried cells.

	Specific activity (counts/min./μmole)			
	Reaction			Control
Equilibration time ...	15 min.	30 min.	60 min.	60 min.
Compound or group				
Carbon dioxide	580	510	508	1330
Pyruvate	282	376	382	0
$-CO_2H$	—	—	394	0
Equilibration (%)	48·6	73·6	75·4	0

It is implicit in mechanism *B* that when lactate is fermented in the presence of labelled carbon dioxide, the carboxyl of the propionate will be labelled and, if $^{14}CO_2$ is used, then the specific activity of the propionate carboxyl will be at least 50 % that of the final carbon dioxide, assuming, of course, that the external carbon dioxide is in equilibrium with the internal carbon dioxide. If the decarboxylation of succinate is reversible, then the specific activity of the propionate will approximate to that of the final carbon dioxide. When lactate was fermented by LC in the presence of $^{14}CO_2$, a minute amount of the isotope was found in the carboxyl groups of acetate and butyrate and the specific activity of the propionate carboxyl group was 6·3 % of that of the final carbon dioxide. The specific activity of the valerate was of the same order as that of the propionate. This value is much lower than that required by theory. Nonetheless, the appearance of even a small amount of carbon dioxide in the propionate has to be explained. Wolfe & O'Kane (1955) have demonstrated a rapid exchange reaction between carbon dioxide and the carboxyl group of pyruvate in extracts of *Clostridium butyricum* (equation 1).

$$CH_3 \cdot CO \cdot CO_2H + {}^{14}CO_2 \rightleftarrows CH_3 \cdot CO \cdot {}^{14}CO_2H + CO_2. \quad (1)$$

This reaction has now been demonstrated in extracts of LC (Table 8). D. J. Walker & J. N. Ladd (in preparation) have shown that in the fermentation of lactate by extracts of LC, part of the lactate is oxidized to pyruvate and, since this reaction is reversible, incorporation of carbon dioxide into the pyruvate by the exchange reaction would lead to the formation of some [1-^{14}C]lactate and hence [1-^{14}C]propionate. When acrylate was the substrate only a minute amount of $^{14}CO_2$ was incorporated into the propionate carboxyl group. No explanation is offered for this difference between the fermentations of lactate and acrylate.

Finally there is no evidence for the decarboxylation of succinate by this organism. Elsden *et al.* (1956) have observed that cells of LC growing on lactate do not utilize succinate added to the medium. Cell-free extracts which fermented lactate rapidly did not decarboxylate succinate even in the presence of adenosine triphosphate and coenzyme A. Nor was it possible to demonstrate an exchange reaction between propionate and carbon dioxide.

These experiments also provide information on the mechanism of the formation of the other volatile fatty acids. The formation of acetate from lactate by the organism LC may be explained by equations 2 and 3. The exclusive labelling of the acetate carboxyl group and the inactivity of the carbon dioxide indicate that they are formed by a direct oxidation of the lactate via pyruvate.

$$CH_3 \cdot {}^{14}CH(OH) \cdot CO_2H \rightarrow CH_3 \cdot {}^{14}CO \cdot CO_2H + 2(H) \quad (2)$$

$$CH_3 \cdot {}^{14}CO \cdot CO_2H + H_2O \rightarrow CH_3 \cdot {}^{14}CO_2H + CO_2 + 2(H). \quad (3)$$

The formation of butyrate from lactate by the organism LC may be explained by equation (4):

$$2CH_3 \cdot {}^{14}CO_2H + 4(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CO_2H. \quad (4)$$

The specific activity of valerate, formed from the fermentation of [2-^{14}C]lactate by washed cells of LC, is twice the specific activity of the added lactate and equal to the sum of the specific activities of the acetate and propionate produced. Valerate, labelled in two ways, theoretically may be formed (equations 5 and 6):

$$CH_3 \cdot {}^{14}CO_2H + CH_3 \cdot {}^{14}CH_2 \cdot CO_2H + 4(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CH_2 \cdot CO_2H \quad (5)$$

$$CH_3 \cdot {}^{14}CH_2 \cdot CO_2H + CH_3 \cdot {}^{14}CO_2H + 4(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot CH_2 \cdot {}^{14}CO_2H. \quad (6)$$

If valerate is synthesized according to equation (5) then the valerate carboxyl group should be unlabelled; if formed according to equation (6) the valerate carboxyl group should be labelled to the extent of 50 % of the total activity of the molecule. Decarboxylation of the valerate shows that the carboxyl group is labelled. The degree of labelling suggests that the acetate carboxyl group contributes exclusively to the valerate carboxyl group. This is confirmed by decarboxylation of the valerate produced by the fermentation of pyruvate plus [1-^{14}C]propionate. The fact that the valerate carboxyl group is now unlabelled shows that none is

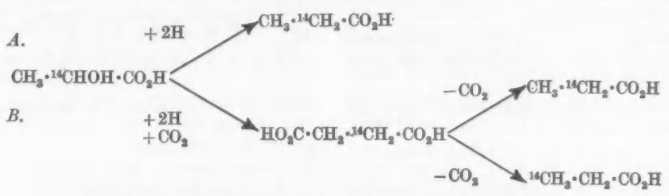

Fig. 1. Formation of labelled propionate from [2-^{14}C]-lactate.

derived from the propionate carboxyl group. These findings are in agreement with those of Stadtman, Stadtman & Barker (1949) working with *Clostridium kluyveri*.

The specific activity of caproate produced from [2-^{14}C]lactate is twice that of the added lactate, suggesting that it is formed by the condensation of two propionate molecules (equation 7):

$$2CH_3\cdot{}^{14}CH_2\cdot CO_2H + 4(H) \rightarrow CH_3\cdot{}^{14}CH_2\cdot CH_2\cdot CH_2\cdot{}^{14}CH_2\cdot CO_2H. \quad (7)$$

If so, the carboxyl group of the caproate should be unlabelled. Decarboxylation of the caproate shows in fact that the carboxyl group is labelled and that it contains approximately 33 % of the activity of the total molecule. These results contradict the specific-activity measurements and suggest that caproate is formed by the condensation of three acetate units (equation 8):

$$3CH_3\cdot{}^{14}CO_2H + 8(H) \rightarrow CH_3\cdot{}^{14}CH_2\cdot CH_2\cdot{}^{14}CH_2\cdot CH_2\cdot{}^{14}CO_2H. \quad (8)$$

This agrees well both with the observations of Elsden & Lewis (1953) that caproate synthesis is increased markedly by the addition of butyrate to washed cells of LC fermenting pyruvate under hydrogen, and with the isotopic data of Stadtman *et al.* (1949) working with *C. kluyveri*. If equation 8 does represent the mechanism, the specific activity of the caproate formed from the fermentation of [2-^{14}C]lactate (Table 1) is too low. This might well be the case, for the yield of caproate was very small (6·5 μmoles in 123 μmoles of total volatile acid) and small absolute errors in the estimation could lead to large relative errors in calculating the specific activity.

SUMMARY

1. The relative specific activities and distribution of label within the products of [2-^{14}C]lactate fermentation by washed cells of the rumen organism LC have been measured. The carbon dioxide produced is unlabelled. The acetate formed is carboxyl-labelled and the propionate is labelled exclusively in the methylene position. The specific activity of the butyrate is twice that of the butyrate carboxyl group; the specific activity of valerate is twice that of the valerate carboxyl group and the specific activity of caproate is three times that of the caproate carboxyl group.

2. The distribution of label within propionate and acetate formed from [2-^{14}C]lactate has been confirmed with cell-free extracts.

3. The specific activity of propionate, formed by the fermentation of lactate in the presence of $^{14}CO_2$ by washed cells of LC, is only 6 % of that of the final carbon dioxide. The propionate formed from acrylate under similar conditions is not significantly labelled.

4. Cell-free extracts of LC catalyse an exchange reaction between the pyruvate carboxyl group and carbon dioxide.

5. The mechanisms of formation of fatty acids from lactate by LC are discussed.

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Transglucosidase Activity of Rumen Strains of *Streptococcus bovis*: Structure of the Dextran Produced from Sucrose

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Many extensive studies have been made on the glucose polymers, known as dextrans, which are produced from sucrose by the transglucosidase activity of species in the genus *Leuconostoc*. Less work has been described, however, on similar polysaccharides produced from sucrose by streptococcal species. Niven, Smiley & Sherman (1941) reported that various strains of *Streptococcus bovis*, of unrecorded origin, produced from sucrose a polysaccharide which yielded glucose on hydrolysis and which was therefore assumed to be dextran. Hehre & Neil (1946) showed, mainly by serological tests, that many strains of '*viridans*' streptococci isolated from the blood of patients with subacute bacterial endocarditis could convert sucrose into a polysaccharide indistinguishable from *Leuconostoc* dextran. *S. bovis* is itself in many respects a '*viridans*' streptococcus (Topley & Wilson, 1946). Jeanes *et al.* (1954) measured, by periodate oxidation, the amounts of main-chain linkages (α-1:6) and branching linkages (α-1:3 and α-1:4) in the dextrans produced by two strains of *Streptococcus viridans*. These two dextrans were reported to contain 11 and 31% of α-1:3, and 4 and 2% of α-1:4-glucosidic linkages respectively. In contrast with these results Barker, Pautard, Siddiqui & Stacey (1955*b*) reported that the dextran produced from sucrose by an unidentified *Streptococcus*, isolated from ropey elderberry wine, contained only α-1:6-glucosidic linkages and was essentially unbranched.

Recently Dain, Neal & Seely (1956) reported that with most strains of *S. bovis* grown on sucrose–agar mucoid colonies containing a glucose polymer were produced only if carbon dioxide was present. The effect of carbon dioxide on the production of this polysaccharide, in liquid sucrose media, by several strains of rumen *S. bovis* has been investigated in detail by Bailey & Oxford (1958). They found that most of the strains failed to produce dextran in such a medium unless carbon dioxide or HCO_3^- ion was present. The polysaccharide appeared to be a dextran containing few, if any, branching linkages. In view of the lack of information on *Streptococcus* dextrans it was decided to investigate, in more detail, the structure of the dextrans produced by the *S. bovis* strains used in the above work. This paper reports the results obtained in a study of the dextrans produced by six strains of rumen *S. bovis* from sucrose at 37° in the presence of carbon dioxide. The effect of incubation temperature and of Mg^{2+} ions in the medium on dextran structure is also briefly described.

EXPERIMENTAL

Streptococcus bovis *strains and cultures.* Six strains were used comprising two (1010Y and 18M2) supplied as freeze-dried cultures by Dr P. N. Hobson and four (A1, A2, I and 293C) freshly isolated from cows at this Laboratory. Full details of all of these strains and of the sucrose (8%, w/v) medium used for the production of dextran are given by Bailey & Oxford (1958). Cultures (100 ml.) were incubated anaerobically for 48 hr. at 37°, CO_2 being provided either as 100% CO_2 gas atmosphere or as HCO_3^- ion (0·01 M).

Isolation and purification of dextran. Crude dextran was precipitated from the cell-free culture fluid with ethanol (2 vol.). Purification of the precipitated dextran was carried out by the usual methods (Barker, Bourne, Bruce, Neely & Stacey, 1954*a*; Bailey & Oxford, 1958). The purified dextran was freeze-dried and finally dried *in vacuo* at 60° over P_2O_5.

Acid hydrolyses. Dextran (20 mg.) in 1·5 N-H_2SO_4 (10 ml.) was heated for 9 hr. at 100°, neutralized (3 N-NaOH) and diluted to 100 ml. with water. Reducing sugars in the hydrolysate were measured by the cuprimetric method of Shaffer & Hartmann (1921) and the results, corrected by the factor of Pirt & Whelan (1951), reported as glucose. For paper-chromatographic analyses the acid hydrolysate was neutralized with solid $BaCO_3$, filtered and concentrated to 0·2 ml. Partial acid hydrolyses were carried out by heating the dextran–acid mixture (above) at 100° for 2 hr.

Paper-chromatographic analyses. Chromatograms were prepared and developed by the usual techniques with a solvent consisting of the top layer of either: (*a*) butanol-1–ethanol–water–aq. NH_3 soln., density 0·91 (49:10:40:1, by vol.; Barker *et al.* 1954*a*) or (*b*) ethyl acetate–water–pyridine (2:2:1, by vol.; Jermyn & Isherwood, 1949). Sugars were detected with $AgNO_3$ (Trevelyan, Procter & Harrison, 1950), aniline hydrogen phthalate (Partridge, 1949) and benzylamine–ninhydrin (Bayly & Bourne, 1953).

Periodate oxidations. Dextran (100 mg.) dissolved in water (250 ml.) containing 5 ml. of 0·3 M-sodium metaperiodate was incubated in the dark at 28°. Periodate consumption and formic acid production were measured at

24 hr. intervals by the methods described by Jeanes & Wilham (1950). After 120 hr. the oxidized dextran solution (80 ml.) was treated with ethylene glycol (1 ml.), dialysed against tap water and concentrated under vacuum to a small volume (5 ml.). The concentrate was hydrolysed with 3N-H_2SO_4 (5 ml.) at 100° for 9 hr. and the hydrolysate neutralized and concentrated to a syrup for paper-chromatographic analysis.

Optical rotations. These were measured with a Hilger polarimeter in a 1 dm. tube containing dextran (20–40 mg.) dissolved in N-NaOH (10 ml.).

Infrared spectra. Spectrograms were obtained over the range 700–900 cm.$^{-1}$ on a Perkin–Elmer, model 21, double-beam infrared spectrophotometer with Nujol (liquid paraffin) films of the dextrans.

Authentic Leuconostoc *dextran.* A specimen of *Leuconostoc* dextran, prepared by the author in Professor M. Stacey's Laboratory, Birmingham University, was used for comparative purposes and as a source of authentic isomaltose and isomaltotriose. This dextran, produced from sucrose at 25° by *Leuconostoc arabinosaceus* (*Betacoccus arabinosaceus*, Birmingham strain), contained about 15% of α-1:3 linkages but no α-1:4 linkages (Barker *et al.* 1954*a*).

RESULTS

Yields and analyses of the dextrans produced by strains of Streptococcus bovis. The yield of purified dextran produced by each strain of *S. bovis* (as reported by Bailey & Oxford, 1958) together with the optical rotation and glucose content of the dextran is listed in Table 1. Results obtained from the periodate oxidation of the dextrans, after oxidation for 96 hr., are also listed in Table 1. Periodate consumption and formic acid production showed no change after 96 hr. Acid hydrolysates of the final, concentrated, oxidized dextran solutions showed on paper chromatograms only a trace component corresponding to glucose. The amount of glucose present on the papers from all six oxidized dextrans was estimated, by visual comparison with standards, to correspond to less than 0·5% of the original dextran. In contrast with these results oxidized *Leuconostoc* dextran hydrolysate showed on the papers a strong glucose component corresponding to 10–15% of the original dextran.

Effect of cultural conditions on dextran structure. S. bovis (strain I) was grown in the standard medium at 28°. At this temperature growth was slower and the yield of dextran only 65% of that at 37°. Strain I was also grown at both 28° and 37° in a modified medium containing in addition magnesium acetate (0·008 M-Mg^{2+} ions). Both the dextrans produced at 28° were noticeably less soluble in water than any of the dextrans produced at 37°. All three dextrans had the same optical rotation as the strain I dextran listed in Table 1 and consumed after oxidation for 96 hr. 1·99–2·00 moles of periodate and produced 0·98–0·99 mole of formic acid/mole of anhydroglucose. Only trace amounts (less than 0·5%) of glucose were detected in acid hydrolysates of the oxidized dextrans.

Infrared spectra of the dextrans. Spectrograms prepared from dextran from each strain of *S. bovis* showed two absorption peaks at 760–765 cm.$^{-1}$ and at 842–844 cm.$^{-1}$ Barker, Bourne, Stacey & Whiffin (1954*b*) record three absorption peaks for dextrans in this region at 841, 794 and 768 cm.$^{-1}$ and assign them to the α configuration, α-1:3-glucosidic linkage and α-1:6-glucosidic linkage respectively. The second of these three absorption peaks was absent from the spectrograms of all six *Streptococcus* dextrans but present on that of the *Leuconostoc* dextran.

Paper-chromatographic analyses of dextran hydrolysates. Total acid hydrolysates of all six *Streptococcus* dextrans showed on the papers a single reducing component chromatographically identical with glucose. Partial acid hydrolysates of each dextran showed components corresponding to glucose and an apparently homologous series of oligosaccharides (detected with silver nitrate or aniline hydrogen phthalate). The first two of these oligosaccharides were chromatographically identical with authentic isomaltose and isomaltotriose on papers developed with either solvent (*a*) or (*b*) or by the benzylamine–ninhydrin technique. No sign of the disaccharides derived from possible branch linkages (nigerose or maltose) was seen on any of the papers.

Table 1. *Yields and analyses of dextrans produced by rumen strains of* Streptococcus bovis *in liquid sucrose media in the presence of carbon dioxide at* 37°

S. bovis strain no.	Dextran yield (% conversion of available glucose)	Glucose content (%)	$[\alpha]_D^{22}$ (in N-NaOH)	Moles of periodate consumed/mole of dextran anhydroglucose	Moles of formic acid produced/mole of dextran anhydroglucose
1010Y	37·9	96·0	+187	1·94	0·97
18M2	83·5	99·1	+189	2·00	0·95
A1	17·9	96·05	+190	1·97	0·98
A2	67·3	96·50	+194	2·00	0·98
I	50·8	97·0	+190	2·00	0·96
293C	27·2	97·0	+192	1·99	1·005
Leuconostoc dextran	—	96·0	+202	1·67	0·74

Oligosaccharides isolated from a dextran hydrolysate. Dextran (strain I; 1·5 g.) dissolved in 1·5N-H_2SO_4 (100 ml.) was heated for 2 hr. at 100°, and the hydrolysate, after neutralizing ($BaCO_3$) and filtering, was adsorbed on a charcoal–Celite column (40 cm. × 3·5 cm.; Whistler & Durso, 1950). Sugars were eluted from the column with water and water–ethanol mixtures. After removal of glucose (water 3 l., followed by ethanol–water, 2%, v/v; 2 l.) chromatographically pure disaccharide was eluted with ethanol–water, 5%, v/v (1 l.). Stronger ethanol solution (7·5%, v/v; 1 l.) eluted a trisaccharide fraction. The two fractions were concentrated under vacuum and freeze-dried, then treated with anhydrous methanol to remove inorganic material, and finally freeze-dried again and vacuum-dried. The yield of disaccharide was 200 mg. of glassy solid and of trisaccharide 150 mg. of solid. The disaccharide showed on paper chromatograms a single component chromatographically identical with authentic isomaltose, the trisaccharide showed a single component identical with authentic isomaltotriose. No sign of any other disaccharide was detected in any of the washings from the column.

The disaccharide had $[\alpha]_D^{20} + 120°$ (*c*, 0·20, in water); Montgomery, Weakly & Hilbert (1949) report $[\alpha]_D^{25} + 120°$ for isomaltose. The disaccharide (70 mg.) was acetylated with acetic anhydride and sodium acetate (Barker *et al.* 1954*a*), and worked up in the usual manner and crystallized from ethanol to yield 60 mg. of crystalline solid. The crystalline acetate had m.p. 142–143° (uncorr.), unchanged by admixture with an authentic sample of β-isomaltose octa-acetate kindly supplied by Dr S. A. Barker, and had $[\alpha]_D^{26} + 100°$ (*c*, 0·4, in chloroform). Barker *et al.* (1954*a*) report for β-isomaltose octa-acetate m.p. 144–145° and $[\alpha]_D^{20} + 99·6°$ (in chloroform).

DISCUSSION

That the glucose polymer produced by *S. bovis* from sucrose is a dextran is clearly established by the identification of isomaltose as the sole disaccharide in partial acid hydrolysates of the polysaccharide. In the absence of methylation studies periodate oxidation is probably the most useful technique for studying dextran structures. An unbranched dextran, containing only α-1:6-glucosidic linkages consumes 2 moles of periodate and produces 1 mole of formic acid/mole of anhydroglucose. Branch linkages, either α-1:3 or α-1:4, cause a significant lowering of both periodate consumption and formic acid production. The presence of free glucose in the hydrolysate of the oxidized dextran is also a useful confirmatory indication of the presence of α-1:3-glucosidic linkages in the original dextran. The values obtained for periodate consumption and formic acid production with all six *S. bovis* dextrans clearly indicate the absence of more than traces of α-1:3 or α-1:4 linkages. The absence of α-1:3 linkages is confirmed by the low level (less than 0·5%) of glucose in the oxidized dextrans, and by the infrared spectra.

Highly branched dextrans would show disaccharides other than isomaltose (i.e. nigerose or maltose) on paper chromatograms of their partial hydrolysates (see Barker *et al.* 1954*a*). These other disaccharides were never detected. Specific optical rotations (+187 to +194°) confirm that the glucosidic linkages have an α configuration and are in the same range as values reported for other unbranched dextrans; e.g. unbranched *Streptococcus* dextran +194° (Barker *et al.* 1955*b*), unbranched *Leuconostoc* dextran +194° (Barker, Bourne, James, Neely & Stacey, 1955*a*). The specimen of *Leuconostoc* dextran, containing about 15% of α-1:3-glucosidic linkages, had $[\alpha]_D^{20} + 202°$ (in N-sodium hydroxide). Higher values, up to 220°, have been reported for branched dextrans of this type (Bailey, Barker, Bourne & Stacey, 1957).

All of the results obtained indicate that the dextrans produced by six strains of rumen *S. bovis* are unbranched polysaccharides containing only α-1:6-glucosidic linkages. The structures of the dextrans do not appear to reflect any differences between the strains.

It is possible that incubation temperature has an effect on the branching of dextrans. Both Barker *et al.* (1955*b*) and Jeanes *et al.* (1954) grew their streptococci at 25°. Working with a *Leuconostoc* dextransucrase, Bailey *et al.* (1957) reported that the enzyme producing the branch linkage (α-1:3) was much less stable, even at 0°, than the enzyme producing the main chain (α-1:6). Our streptococci were grown at 37°; the temperature of the rumen, the organism's natural habitat, is just below 40°. When *S. bovis* (strain I) was grown at a lower temperature (28°) the dextran produced was still unbranched. Barker *et al.* (1955*a*) also reported that magnesium was essential for the production of a highly branched dextran by one strain of *Leuconostoc*. The medium used in the present work would contain traces of magnesium. When the medium was enriched with magnesium *S. bovis* (strain I) still produced an unbranched dextran at both 28° and 37°.

Under the cultural conditions used in the present work it seems that rumen strains of *S. bovis* are useful organisms for the synthesis of unbranched dextran. They may also prove a useful source of a dextransucrase free from enzymes which synthesize α-1:3- and α-1:4-glucosidic linkages. The properties of the dextransucrase secreted by *S. bovis* are being investigated.

SUMMARY

1. The polysaccharide produced by rumen strains of *Streptococcus bovis* from sucrose at 37°, in the presence of carbon dioxide, yielded only glucose on hydrolysis.

2. Isomaltose was isolated and identified as the sole disaccharide produced by partial acid hydrolysis of the polysaccharide, which is therefore a dextran.

3. On the evidence of periodate oxidation, infrared spectra and optical rotations it is concluded that the dextrans produced by six strains of *S. bovis* contain only α-1:6-glucosidic linkages and are therefore unbranched.

4. Growth of one strain of *S. bovis* at a lower temperature (28°) or in a medium containing added magnesium still produced an unbranched dextran.

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The Anaerobic Metabolism of Citrate in Rat Liver

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In experiments with washed particles of rat liver, Vignais, Vignais & Bartley (1957) showed that under aerobic conditions the uptake of oxygen was insufficient to account for the removal of tricarboxylic acids. This paper is concerned with the examination of the products of anaerobic citrate metabolism in washed particulate preparations of rat liver.

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EXPERIMENTAL

Washed particles. Rats were stunned and beheaded and the livers were rapidly removed and chilled in ice. After removal of surplus ice the livers were minced in the Fischer mincer (Jouan, Paris). The pulp from one liver (8–10 g.) was homogenized with 30 ml. of 0·9% KCl soln. in a stainless-steel homogenizer of the Potter–Elvehjem type. The homogenate was made to 60 ml. with 0·9% KCl soln., and the particulate material that sedimented after centrifuging at 0° for 15 min. at 2000 *g* was collected and washed twice on the centrifuge with 30 ml. of 0·9% KCl, the same centrifugal conditions being used for both washings.

This mixture of washed nuclei and mitochondria is similar to that used by Green, Loomis & Auerbach (1948) and named by them 'cyclophorase'. Because it is very quickly and easily prepared 'cyclophorase' was used for all the experiments illustrated in this paper, but other unpublished work indicates that mitochondrial preparations isolated from 0·25 M-sucrose according to Werkheiser & Bartley (1957) consumed citrate at about the same rate as the 'cyclophorase' preparations.

Special chemicals. ^{14}C-Labelled citrate was a gift from Dr H. L. Kornberg. It had been prepared by incubation of ^{14}C-labelled acetate with oxaloacetate in the presence of a bacterial extract which contained condensing enzyme. The products of the reaction were separated on a paper chromatogram with the solvents of Kornberg (1958) and the citrate area was eluted with water. It is not completely certain that the ^{14}C-labelled citrate was entirely free from other compounds. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; other chemicals were commercial products, of A.R. standard when available.

Incubation procedure. To the main compartment of each Warburg vessel standing in cracked ice was added 2 ml. of particles (usually about 100 mg. dry wt.) suspended in 0·9% KCl and the other additions which are described in the individual experiments. The citrate was added to the side arm and the centre well contained a stick of yellow phosphorus. The final fluid volume was 4 ml. When the vessels were attached to the manometers they were placed in a rack so that the vessels were immersed in an ice–water bath. Each vessel was gassed for at least 5 min. with O_2-free N_2 and then left to stand in the ice–water bath for 15 min. with the taps closed. During this time the vessels were gently shaken to ensure thorough anaerobiosis. The contents of the side arm were then mixed and the manometers were shaken in a water bath at 40°. The manometers were removed from the bath at various times and the reaction was stopped by the addition of 0·5 ml. of 30% trichloroacetic acid to the vessel. After removal of the precipitated protein by centrifuging the supernatant fluid was stored at −18° until it could be analysed.

Enzyme preparations. Fumarase (Massey, 1952) was purified to the stage of the first $(NH_4)_2SO_4$ fractionation. Aconitase was either the crude preparation of Krebs & Eggleston (1944) or later the purified preparation of Morrison (1954) taken as far as the first $(NH_4)_2SO_4$ fractionation stage. Succinoxidase was prepared from pigeon-breast muscle as described by Krebs (1937). TPN *iso*citric dehydrogenase was prepared according to Ochoa (1948).

Chemical estimations. Succinate was measured by an unpublished method of Bartley & Notton involving conversion into malate with succinoxidase and subsequent fluorimetric estimation of the malate (Hummel, 1949). β-Hydroxybutyrate was estimated by the method of Greenberg & Lester (1944) as modified by R. G. Kulka (unpublished work). Malate was estimated either with *Lactobacillus arabinosus* by the method of Nossal (1951) or fluorimetrically as described by Hummel (1949). Carbohydrate was hydrolysed to glucose and estimated according to Miller & Van Slyke (1936). Other chemical estimations were by the following methods: citrate, Taylor (1953); α-keto acids, Friedemann & Haugen (1943); total amino acids, Rosen (1957); acetate, Serlin & Cotzias (1955); lactate, Barker & Summerson (1941).

Tissue dry weights. A measured volume of the tissue suspension was dried at 105° for 24 hr. and weighed. In calculation of tissue activities no correction was made for the KCl content of the suspensions.

Paper chromatography. 2:4-Dinitrophenylhydrazones of α-keto acids were separated according to El Hawary & Thompson (1953); organic acids by two-dimensional chromatography according to Kornberg's (1958) modifications of the method of Calvin & Benson (1949). Radioautographs were made as described by Kornberg (1958) and the radioactivity was counted on the chromatograms with a large end-window counter (5 cm. diameter).

Reproducibility. Normally the data were derived from chemical analysis of the contents of a single Warburg vessel and from measurements of gas changes made in the vessel during incubation. Sufficient preliminary work was done to show that the gas output in duplicate vessels incubated for the same time and under the same conditions was the same. In the chemical determination of tricarboxylic acid duplicate determinations were made. These always agreed by better than 5%.

RESULTS

Comparison of the aerobic and anaerobic metabolism of tricarboxylic acids by rat-liver particles. Table 1 shows the comparative consumption of citrate by washed particles of rat liver under anaerobic or aerobic conditions. The anaerobic consumption of tricarboxylic acid is approximately one-half of that consumed aerobically. The Q_{O_2} of the preparation was 35 (μl. of O_2/mg. dry wt./hr.). Thus the ratio, O_2 consumption/tricarboxylic acid consumption (1·5/0·88), indicates that the citrate consumed

Table 1. *Comparison of the aerobic and anaerobic metabolism of citric acid by rat-liver particles*

Warburg vessels contained 131 mg. dry wt. of particles added in 2·5 ml. of 0·9% KCl soln., 0·2 ml. of 5 mM-ATP, 0·2 ml. of 5 mM-$MgCl_2$, 0·2 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, and 1·4 ml. of 0·9% KCl; 0·1 ml. of 0·5 M citrate was added from the side arm. The reaction was stopped by the addition of 0·5 ml. of 30% trichloroacetic acid. Samples of the extracts were analysed for citric acid after the action of aconitase.

Incubation time (min.)	Total tricarboxylic acid content of vessels (μmoles)		Tricarboxylic acid consumption (μmole/mg. dry wt./hr.)	
	Anaerobic	Aerobic	In N_2	In O_2
0	72	72	—	—
30	43·9	11·7	0·38	0·88

was not being completely oxidized, but that the oxidation proceeded past the stage of α-oxoglutarate (see Vignais *et al.* 1957).

Effect of the tissue concentration on anaerobic formation of carbon dioxide from citrate and citrate removal. Although more tricarboxylic acid was consumed when the amount of tissue was increased the amount reacting was not proportional to the amount of tissue. The ratio of CO_2 production to tricarboxylic acid consumed was greater with higher concentrations of tissue (Table 2), and reached values above 1·0. Two reactions may be expected to occur (Stern, Shapiro, Stadtman & Ochoa, 1951) whereby citrate is metabolized:

$$\text{Citrate} + \text{coenzyme A} \rightarrow \text{oxaloacetate} + \text{acetyl coenzyme A} \quad (1)$$

$$\text{Citrate} \rightarrow \textit{iso}\text{citrate} \rightarrow \alpha\text{-oxoglutarate} + CO_2 + 2H. \quad (2)$$

Reaction (2) is dependent on a supply of TPN and DPN and on an acceptor for the hydrogens removed from the *iso*citrate. On the other hand, the dissimilation reaction (1) can occur without these coenzymes. It is possible that the increase of tissue increases the ratio CO_2 produced/tricarboxylic acid consumed by contributing a greater amount of endogenous cofactors.

Effect of citrate concentration. As shown in Table 3 an increase of citrate concentration resulted in a lowering in the ratio CO_2 production/tricarboxylic acid consumption.

Effect of di- and tri-phosphopyridine nucleotide and adenosine triphosphate on citrate consumption and carbon dioxide production. Table 4 shows the effect of DPN, TPN and ATP on the anaerobic citrate consumption and on CO_2 production. When added separately both DPN and TPN stimulated the removal of citrate to the same extent (Table 4), but TPN stimulated CO_2 production more than DPN. ATP alone gave a small increase in consumption of tricarboxylic acid but a large increase in CO_2 production. When TPN and ATP were added together the increase in CO_2 and tricarboxylic acid removal were larger than with TPN alone and there was an increase in the ratio CO_2 production/citrate consumption. When DPN and ATP were added together an increase in citrate consumption and CO_2 production occurred but the ratio CO_2 produced/citrate consumed did not change.

Effect of magnesium. When magnesium (mM) was added alone there was a diminution in the tricarboxylic acid removed without alteration in the CO_2 production (Table 4). In the presence of DPN or TPN (Table 4), with or without ATP, magnesium had various effects; thus when added with DPN it slightly increased the removal of tricarboxylic acid and increased the CO_2 production, but when ATP was also present the consumption of tricarboxylic acid was reduced and the CO_2 production was unaltered. In the presence of TPN magnesium did not alter the consumption of tricarboxylic acid but reduced CO_2 production. When ATP was present, together with the TPN, there was virtually no effect of magnesium.

Effect of dithionite, hydroxylamine and ethylenediaminetetra-acetic acid. Dithionite might be expected to inhibit any reactions involving dehydrogenations, as it would reduce all the possible hydrogen acceptors. As shown in Table 5, this in fact did occur, as the addition of dithionite caused a marked diminution in the tricarboxylic acid consumed and an even greater drop in the CO_2 evolved. The addition of hydroxylamine had only a slight effect in reducing consumption of tricarboxylic acid but did reduce the CO_2 production by about 30%. It was unexpected that the addition of ethylenediaminetetra-acetic acid (EDTA) should also reduce the consumption of tricarboxylic acid. This effect of EDTA was not seen when the incubation medium contained magnesium chloride.

Table 2. *Effect of the amount of tissue on the anaerobic citrate consumption and carbon dioxide production by washed particles of rat liver*

Warburg vessels contained 0·5 ml. of 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4, and washed particles of rat liver (amounts indicated) suspended in 0·9% KCl. The side arm contained 0·1 ml. of 0·5 M-sodium citrate. The final volume in each flask was made to 4 ml. with 0·9% KCl. Gas phase was N_2, the incubation temperature 40° and the incubation time 2 hr. The reaction was stopped by the addition of 0·5 ml. of 30% (w/v) trichloroacetic acid.

Dry wt. of tissue (mg.)	Output of CO_2 (μmoles)	Consumption of tricarboxylic acid (μmoles)	Ratio, CO_2 produced/ tricarboxylic acid consumed
31·6	1·9	7·0	0·27
63·4	6·0	8·7	0·69
126·8	10·3	14·0	0·74
253·6	24·4	22·7	1·07

Table 3. *Effect of the amount of citrate on the anaerobic citrate consumption and carbon dioxide production by washed particles of rat liver*

Warburg vessels contained 2 ml. of washed partieles of rat liver (152 mg. dry wt.) suspended in 0·9% KCl and 0·5 ml. of 0·1 M-tris buffer, pH 7·4; 0·1 M-sodium citrate was added from the side arms in the volumes shown below. Other details are as described in Table 2.

Vol. of citrate (ml.)	Output of CO_2 (μmoles)	Consumption of tricarboxylic acid (μmoles)	Ratio, CO_2 produced/ tricarboxylic acid consumed
0	0·62	—	—
0·05	6·90	4·29	1·61
0·10	7·15	4·65	1·54
0·20	12·58	8·60	1·46
0·50	16·30	14·90	1·09

Effect of cyanide on the consumption of tricarboxylic acid. The addition of cyanide (0·01 M) completely inhibited the disappearance of citrate.

Effect of phosphate on the consumption of tricarboxylic acid. Substituting phosphate buffer for 2-amino-2-hydroxymethylpropane-1:3-diol (tris) had no effect on the disappearance of tricarboxylic acid.

Products of the anaerobic metabolism of citrate. Malate and α-oxoglutarate accumulated on incubation of citrate with liver (Table 6). These two substances probably arise by the oxaloacetate

Table 4. *Effect of magnesium, adenosine triphosphate, diphosphopyridine nucleotide and triphosphopyridine nucleotide on the anaerobic citrate consumption and carbon dioxide production of washed particles of rat liver*

Incubation conditions were as described in Table 3. Dry wt. of tissue/vessel in Expts. 1 and 2 = 79·2 mg., and in Expt. 3 = 111·6 mg.

Expt. no.	Addition	Amount of addition (μmoles)	Output of CO_2 (μmoles)	Consumption of tricarboxylic acid (μmoles)	Ratio, CO_2 produced/ tricarboxylic acid consumed
1	None	—	8·0	12·3	0·65
	TPN	1·3	13·5	13·9	0·97
	TPN + $MgCl_2$	1·3 4·0	9·8	13·9	0·71
	TPN + ATP	1·3 4·0	15·0	16·3	0·92
	TPN + $MgCl_2$ + ATP	1·3 4·0 4·0	15·5	15·7	0·99
2	None	—	10·6	10·1	1·05
	ATP	4·0	17·8	12·3	1·45
3	None	None	13·6	12·0	1·13
	$MgCl_2$	4·0	13·7	7·2	1·90
	DPN	1·5	17·5	13·9	1·26
	$MgCl_2$ + DPN	4·0 1·5	15·3	14·8	1·03
	DPN + ATP	1·5 4·0	21·0	18·3	1·15
	$MgCl_2$ + DPN + ATP	4·0 1·5 4·0	21·4	16·5	1·29

Table 5. *Effect of dithionite, hydroxylamine and ethylenediaminetetra-acetic acid on the anaerobic citrate consumption and carbon dioxide production of washed particles of rat liver*

Incubation conditions were as described in Table 2. Dry wt. of tissue, 96·2 mg./vessel.

Addition	Amount of addition (μmoles)	Output of CO_2 (μmoles)	Consumption of tricarboxylic acid (μmoles)	Ratio, CO_2 produced/ tricarboxylic acid consumed
None	—	10·5	10·1	1·04
Dithionite	30	4·0	6·6	0·61
Hydroxylamine	40	7·0	9·1	0·77
Ethylenediaminetetra-acetic acid	20	8·0	6·6	1·23

Table 6. *Formation of malate plus fumarate and α-oxoglutarate during the anaerobic metabolism of citrate*

Incubation conditions were as described in Table 2 except that in Expt. 3 incubation was for 1 hr. Expt. 2 is the same washed particle preparation as in Expt. 3 but it was incubated for the full time of 2 hr.

Expt. no.	Consumption of tricarboxylic acid (μmoles)	Formation of malate + fumarate (μmoles)	Formation of α-oxoglutarate (μmoles)	Percentage of citrate recovered as malate + fumarate + α-oxoglutarate
1	7·6	1·2	0·34	20
2	14·2	2·1	0·55	18
3	7·6	1·4	1·30	36
4	9·4	1·9	0·30	23

formed by (1) acting as hydrogen acceptor when *iso*citrate is dehydrogenated by (2). Not more than 36% of the citrate disappearing was accounted for in these two compounds and the amount of α-oxoglutarate found was always less than the sum of malate and fumarate. Thus it appears that either citrate is metabolized by a pathway other than that of the citric acid cycle or the malate and α-oxoglutarate are further metabolized. One conceivable pathway that has not yet been demonstrated in mammalian tissue is the cleavage of *iso*citrate to give glyoxalate and succinate (*iso*-citritase: see Saz & Hillary, 1956). However, no glyoxalate was ever detected as a product of the reaction as tested for by the addition of 2:4-dinitrophenylhydrazine and chromatography of the hydrazones on paper (see also Madsen, 1958). Further, no radioactivity appeared in glyoxalate when radioactive citrate was incubated anaerobically in the presence of glyoxalate. This possibility was not therefore pursued but tests were made for the products of the further metabolism of α-oxoglutarate and malate.

Formation of glutamic acid. α-Oxoglutarate in the presence of ammonium salts is rapidly reduced to glutamate by liver (Krebs, Eggleston & Hems, 1948). Preliminary tests showed that the incubation of rat-liver particles with citrate under anaerobic conditions was accompanied by the appearance of a ninhydrin-reactive substance which ran on the paper chromatogram in the same position as glutamic acid. However, glutamic acid was also produced on the incubation of liver without added citrate. Thus the incubation of 150 mg. dry weight of particles with 56 μmoles of citrate anaerobically for 2 hr. resulted in the formation of 12·7 μmoles of glutamate. Without citrate 3·6 μmoles of glutamate were formed. In another experiment radioactive citrate was incubated with rat liver anaerobically and the products of the reaction were isolated on the paper chromatograms and tested for radioactivity. About 30% of the counts that were lost from the citrate appeared in the glutamate spot. However, it is still not certain that all the excess of glutamate formed in the presence of the relatively high citrate concentrations was derived from the citrate. The amount of glutamate formed in the absence of citrate was variable, sometimes amounting to as much as 8–10 μmoles/150 mg. dry weight of tissue/2 hr. When there was a high endogenous formation of glutamate the addition of citrate produced a comparatively small increase in the glutamate which accumulated. Paper chromatograms did not show a generalized increase in all amino acids but only in glutamate (sometimes a small amount of aspartate was seen); moreover, when the total increase in ninhydrin-reacting substances was measured this was approximately equal to the glutamate formed as measured by the *Clostridium welchii* decarboxylase. It therefore appears unlikely that the endogenous glutamic acid arises from the breakdown of protein.

Succinate. The sum of malate plus fumarate, α-oxoglutarate and glutamate accounted for only about 50–60% of the citrate disappearing from the incubation medium. Incubation of a rat-liver cyclophorase preparation with ^{14}C-labelled citrate and subsequent paper-chromatographic separation of the organic acids showed that about 40% of the counts disappearing from citrate appeared in an acid which cochromatographed with succinate. Subsequent analysis of further liver preparations incubated with non-isotopic citrate showed that succinate was a consistent product of the reaction.

Other products. On incubation in the absence of citrate the endogenous carbohydrate decreased and there was an increase in endogenous lactate. Incubation with citrate produced an increase in carbohydrate but greatly stimulated lactate production. For example, in a typical experiment incubation without substrate produced an increase in lactate of 0·44 μmole, whereas in the presence of citrate the increase was 1·7 μmoles. In the case where the determination was made there was a small increase in β-hydroxybutyric acid on adding citrate.

If reaction (1) is to continue acetyl-coenzyme A must be hydrolysed and acetate would be expected to accumulate. Tests showed that acetate did accumulate in amounts about 1·5 times that of the malate plus fumarate that was found. These findings imply the presence in the liver of deacylases active in hydrolysing acetyl-coenzyme A.

Recovery of citrate disappearing as other products. It was not technically feasible to estimate all the possible products of anaerobic citrate metabolism in a single experiment. Therefore the results of many experiments have been combined and the yield of each compound has been expressed as the average percentage of the citrate disappearing in each individual experiment (Table 7). It appears probable from these results that most of the citrate disappearing anaerobically (80%) has been

Table 7. *Products of the anaerobic metabolism of citrate*

The average percentage (on a molar basis) of citrate consumed that appeared in the products given below is shown. Other compounds include lactate, β-hydroxybutyrate and carbohydrate.

Compound	Percentage of citrate	No. of experiments
Malate	10	7
α-Keto acids	4	9
Glutamic acid + glutamine	32	11
Succinate	34	6
Other compounds	19	8

accounted for as the sum of malate plus fumarate, keto acids, glutamic acid plus glutamine and succinate. The remaining 20% is apparently accounted for by carbohydrate, lactate and β-hydroxybutyrate. However, the number of estimations done on these three substances are not sufficient to be certain of the recovery, particularly as the percentage is heavily weighted by the contribution of carbohydrate, the formation of which varied greatly.

DISCUSSION

The rate of oxygen uptake by washed particulate preparations of liver in the presence of citrate or *iso*citrate is only about one-half of the rate that occurs with other members of the tricarboxylic acid cycle with the exception of malate. In unfractionated homogenates of rat liver citrate is oxidized at about 80% of the rate of the other acids, with the exception of malate or fumarate which are oxidized at a rate that is only some 50–60% of that of the other components (L. V. Eggleston unpublished work). The lowered rate of respiration in the presence of citrate on fractionation of the homogenate is probably due to the removal of the soluble *iso*citric dehydrogenase. It is generally agreed that the slow rate of oxidation of malate is due to the accumulation of oxaloacetate.

Under anaerobic conditions the occurrence of the following reactions is visualized:

Citrate + coenzyme A ⇋ oxaloacetate + acetyl-coenzyme A (*a*)

Acetyl-coenzyme A → acetate + coenzyme A (*b*)

Oxaloacetate + 2H ⇋ malate ⇋ fumarate (*c*)

*iso*Citrate − 2H → α-oxoglutarate + CO_2 (*d*)

α-Oxoglutarate − 2H → succinate + CO_2 (*e*)

α-Oxoglutarate + 2H + NH_3 ⇋ glutamate (*f*)

Oxaloacetate → pyruvate + CO_2 (*g*)

Pyruvate + 2H ⇋ lactate (*h*)

Fumarate + 2H ⇋ succinate (*i*)

The amount of oxaloacetate formed depends on the extent of reaction (*b*). The reversal of (*b*) requires the participation of ATP and under the anaerobic conditions of the experiments the level of ATP and other nucleotide triphosphates will be very low because of lack of oxidative phosphorylation and because the adenosine triphosphatase activity of the tissue preparations is high. The coupling of reactions (*c*) and (*d*) would lead to the formation of α-oxoglutarate. Further coupling of (*d*) and (*f*) allows the accumulation of glutamate from *iso*citrate in the presence of only catalytic amounts of α-oxoglutarate provided that a source of ammonia is available.

Source of ammonia and the formation of glutamic acid in the absence of substrate. It seems clear that the ammonia for the formation of glutamic acid from *iso*citrate does not arise by transamination from other amino acids since there was no corresponding increase in α-keto acids in the medium. The total amount of glutamine available has not been measured but the endogenous content of glutamic acid plus glutamine was between only one-third and one-twentieth of the glutamic acid that was formed during incubation with citrate, and the glutamic acid plus glutamine that is liberated from endogenous sources on incubation in the absence of citrate is only one-half to one-seventh of the extra glutamic acid that is formed on incubation with citrate. Thus the apparent amounts of glutamine that are probably available fall considerably short of the amount of amino nitrogen that would be required for the observed formation of glutamate.

In most of the experiments ATP was added to the incubation medium. The possibility of this ATP acting as the ammonia donor was not directly tested but the following observations argue against it. The formation of glutamic acid was usually much greater than the 4 μmoles of ATP added and glutamic acid was still formed when ATP was omitted from the incubation medium. Further, as judged by visual inspection of chromatograms that were run to separate the phosphate compounds of the incubated mixture, almost all of the added ATP was recovered as adenosine monophosphate. The formation of glutamate anaerobically from α-oxoglutarate in homogenates has been described by Krebs *et al.* (1948), who also described the stimulating effect of citrate on this reaction. This paper extends these observations and shows that glutamic acid formation can occur in washed particulate preparations without the addition of ammonia and that substantial quantities of glutamic acid can be formed from endogenous sources.

Effect of coenzymes. The addition of DPN, TPN and ATP all increased the output of carbon dioxide from the preparations, as might be expected if they stimulated the *iso*citric enzyme (TPN), α-oxoglutarate oxidase (DPN + ATP) and glutamic dehydrogenase (DPN or TPN).

Effect of ketone-binding substances. It would be expected that ketone-binding substances would inhibit the decarboxylation reactions involved by stabilizing the keto group adjacent to the carboxyl group undergoing decarboxylation. It was not expected that cyanide would completely inhibit citrate removal. This is probably because oxaloacetic cyanhydrin forms sufficiently fast to prevent any appreciable accumulation of oxaloacetate and α-oxoglutarate and thus these compounds are not available to accept the hydrogen from *iso*citrate.

Effect of ethylenediaminetetra-acetic acid. This probably functions by chelating the Mn^{2+} ions necessary for the *iso*citric enzyme (see Moyle & Dixon, 1956).

Little attention appears to have been paid to the anaerobic metabolism of the citric acid cycle intermediates by mitochondrial and other washed particulate preparations. It is perhaps surprising to find such a high consumption of tricarboxylic acid under strictly anaerobic conditions by these preparations since the mitochondria are the sole site of the terminal oxidase of the electron-transport chain. The extensive oxidoreductions that can occur under anaerobic conditions emphasizes the danger of assessing substrate removal by simple measurement of the respiration in its presence since there seems no reason to expect that the competition of the DPN oxidase for the reduced coenzymes would remove them all from interaction with other enzymes and substrates.

SUMMARY

1. Washed particles of rat liver consumed citrate anaerobically at about 50% of the rate occurring aerobically.

2. The products of the anaerobic reaction were on the average malate plus fumarate 10%, α-oxoglutarate 4%, glutamate 32% and succinate 34%. Other substances found were carbohydrate, lactate and β-hydroxybutyrate.

3. The proportions of the substances formed anaerobically from citrate varied with the amount of tissue added and concentration of di- and triphosphopyridine nucleotide and adenosine triphosphate. These three substances increased the citrate consumption whereas dithionite, hydroxylamine and ethylenediaminetetra-acetic acid decreased it and cyanide completely abolished it.

4. Glutamic acid was formed from the tissue preparations on incubation without substrate and was also formed from the citrate added as substrate.

5. The anaerobic reactions of citrate are discussed.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and advice, and Mr L. V. Eggleston for supplying details of his unpublished work. The work was aided by a grant from the Rockefeller Foundation.

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The Identity of Natural and Synthetic ββ-Dimethylacrylylcholine

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The hypobranchial gland of the marine snail *Thais floridana* (Southern oyster drill) has recently been found to contain in relatively high concentration a biologically active ester, identified as ββ-dimethylacrylylcholine (Whittaker, 1957; Keyl, Michaelson & Whittaker, 1957). This paper describes the isolation of the naturally occurring ester as the chemically pure aurichloride, and presents additional evidence for the identity of the natural and synthetic compounds.

The aurichloride of the naturally occurring ester has almost the same melting point and gold content as the synthetic aurichloride and showed no depression of melting point when mixed with the

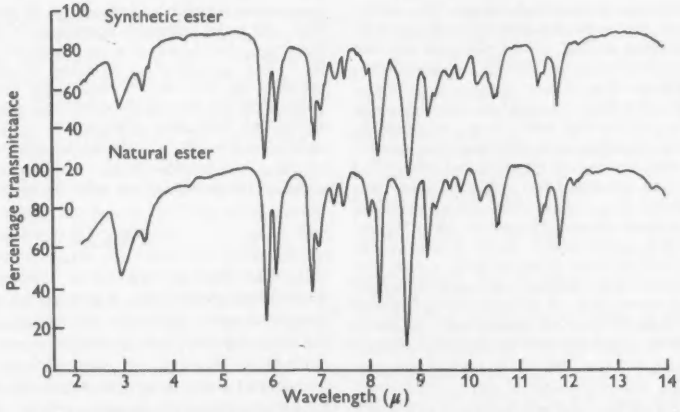

Fig. 1. Infrared-absorption spectra of the aurichlorides of synthetic and natural ββ-dimethylacrylylcholine.

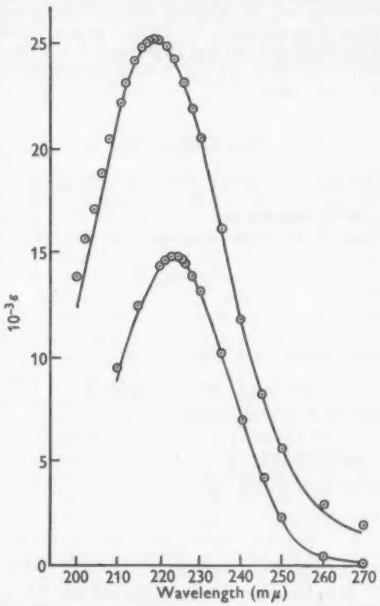

Fig. 2. Ultraviolet-absorption spectra of the aurichlorides (upper curve) and chlorides (lower curve) of synthetic (continuous line) and natural (⊙) ββ-dimethylacrylylcholine. Concentration of esters: 0·025 mM (aurichlorides); 0·05 mM (chlorides).

latter. The infrared (Fig. 1) and ultraviolet (Fig. 2, upper curve) absorption spectra of the two aurichlorides were also almost identical. In the infrared, bonds attributable to a carboxyl group and a C–C double bond in conjugation were detected at wavelengths of 5·84 and 6·03 μ respectively. The C–O–C band at 8·64 μ was also in evidence. The chlorides of the synthetic and naturally occurring esters, obtained by passing the aurichlorides through the anion-exchange resin De-Acidite FF, also had identical ultraviolet spectra (Fig. 2, lower curve), showing the typical absorption band of αβ-unsaturated fatty acid esters with an absorption maximum ($\lambda_{max.}$) at 223 mμ.

EXPERIMENTAL

Preparation of the naturally occurring ester

As aurichloride. Partially purified ester (580 mg.) prepared from snail hypobranchial extracts by reineckate precipitation as described by Keyl *et al.* (1957) was dissolved in water (4·2 ml.), and centrifuged to remove insoluble material and treated with excess of aqueous sodium aurichloride solution. The yellow precipitate was recrystallized twice from hot water, giving yellow needles (48 mg.) with m.p. 94° (Found: Au, 37·4. $C_{10}H_{20}O_2NAuCl_4$ requires Au, 37·5%). The mixed m.p. with the synthetic ester (prepared as described below) was 93–98°.

As chloride. The aurichloride (2·4 mg.) in water (1 ml.) was converted into the chloride by passing it through a column (10 cm. × 0·6 cm.) of De-Acidite FF (The Permutit Co. Ltd., London; −16 + 50 mesh) in the chloride form; 70% of the ester emerged in the first 4 ml. of effluent.

Synthesis of the ester

ββ-Dimethylacrylic anhydride. ββ-Dimethylacrylic acid (27 g., 0·27 mole) was refluxed with acetic anhydride (75 ml., 0·8 mole) for 24 hr. The ββ-dimethylacrylic anhydride was freed from acetic acid and acetic anhydride by repeated distillation *in vacuo*. The yield of light-yellow liquid, b.p. 82·5° at 0·5 mm. Hg, n_D^{25} 1·4866, was 12·5 g. (0·07 mole, 26%).

N-Dimethylaminoethyl-ββ-dimethylacrylate. The above anhydride (12·5 g., 0·07 mole) was refluxed overnight with dimethylaminoethanol (6·25 g., 0·07 mole); the reaction mixture was distilled *in vacuo* to give 15 g. of crude ester, b.p. 60–65° at 0·5 mm. Hg.

ββ-Dimethylacrylylcholine. A mixture of the above crude ester (10 g.) and methyl iodide (20 g., 0·14 mole) in dry ether (125 ml.) was allowed to stand for 3 days at 25°; the precipitate was collected by filtration and crystallized from ethanol to give *ββ-dimethylacrylylcholine iodide* as a light-yellow powder (m.p. 160°), yield, 5 g. (0·015 mole, 32% of dimethylaminoethanol) (Found, C, 38·1; H, 6·5; I, 40·4. $C_{10}H_{20}O_2NI$ requires C, 38·3; H, 6·4; I, 40·5%). The iodide (36 mg., 0·12 m-mole) in water (1 ml.) was converted into the *chloride* by passing it through De-Acidite FF as described above; 75% of the ester emerged in the first 5 ml. of effluent. This was treated with excess of sodium aurichloride to give a yellow precipitate, which on recrystallization from water yielded the ester *aurichloride* as yellow needles, m.p. 97°, yield 12 mg. (0·023 m-mole, 19%) (Found Au, 37·5. $C_{10}H_{20}O_2NAuCl_4$ requires Au, 37·5%). The chloride was also obtained from this salt by passing it through De-Acidite FF as described for the naturally occurring ester.

Spectroscopic comparison of esters

Infrared light. Infrared spectroscopy was carried out in a Perkin–Elmer double-beam recording spectrophotometer with the KBr-window technique. The window contained 0·34% of the ester aurichloride. The spectra of the synthetic and natural esters are plotted side by side in Fig. 1 and will be seen to be essentially identical.

Ultraviolet light. Ultraviolet spectroscopy was carried out in a Hilger Uvispek spectrophotometer. Dilutions were made under standard conditions in relatively large volumes to minimize errors due to absorption of the ester on glassware. All molar-extinction coefficients (ϵ) were calculated from the optical density of a 1 cm. thickness of solution and the molar concentration of ester, determined for the chlorides by the ferric hydroxamate method (Hestrin, 1949) at 520 mμ with the synthetic ester iodide as standard. The results are plotted in Fig. 2. It will be seen that the aurichlorides (upper curve) have a higher $\epsilon_{max.}$ and lower $\lambda_{max.}$ than the chlorides (lower curve), and that the spectra of the synthetic and natural esters are essentially identical.

SUMMARY

1. The biologically active choline ester, ββ-dimethylacrylylcholine, has been isolated from the hypobranchial gland of the marine snail *Thais floridana* as the pure, crystalline aurichloride.

2. The identity of the naturally occurring compound with the synthetic ester is demonstrated by physical and chemical methods.

The synthesis of ββ-dimethylacrylylcholine iodide was carried out by Dr L. E. Tammelin and Ing. I. Enander, and the infrared spectroscopy by Dr L. Larsson, Research Institute of National Defence, Department 1, Sundyberg 4, Sweden. I am most grateful to them for permission to include their results.

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The *in vitro* Enzymic Hydroxylation of Steroids

5. HYDROGEN TRANSPORT IN OX-ADRENOCORTICAL MITOCHONDRIA IN RELATION TO STEROID HYDROXYLATION*

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It has been shown by Grant & Brownie (1955) and by Sweat & Lipscomb (1955) that reduced triphosphopyridine nucleotide (TPNH) is required for the hydroxylation of steroids in the 11β-position by enzymes in ox-adrenocortical mitochondria. Subsequently, Grant (1956*a*) demonstrated that fumarate, added to enzyme reaction mixtures for steroid 11β-hydroxylation, effects triphosphopyridine (TPN^+) reduction according to the reactions: (1) fumarate → L-malate; (2) L-malate + TPN^+ → pyruvate + carbon dioxide + TPNH + H^+. Sweat & Lipscomb (1955) suggested the following sequence of reactions for the formation of TPNH: (3) Fumarate → L-malate; (4) L-malate + diphosphopyridine nucleotide (DPN^+) → oxaloacetate + DPNH; (5) DPNH + TPN^+ → DPN^+ + TPNH. Reaction (5) requires the presence of a pyridine nucleotide transhydrogenase, known to occur in certain animal tissues (Kaplan, Colowick & Neufeld, 1953), but not previously reported in the adrenal cortex. The presence of the transhydrogenase in the adrenal cortex was not demonstrated

* Part 4: Grant (1956*a*).

directly, but was inferred from the observations that when either TPN^+ or DPNH is added to the mitochondrial suspensions in the absence of fumarate or malate only traces of corticosterone are formed from 11-deoxycorticosterone, but when traces of TPN^+ are added with the DPNH a significant amount of 11-deoxycorticosterone 11β-hydroxylation occurs. Both Brownie & Grant (1954) and Sweat & Lipscomb (1955) found that citrate was a relatively feeble 'activator' of steroid 11β-hydroxylation, although its addition to the reaction mixture might be expected to result in reduction of TPN^+ coupled to D-*iso*citrate oxidation. The discovery of a DPN^+-specific *iso*citric dehydrogenase in animal tissues by Plaut & Sung (1954) and the investigations of *iso*citrate oxidation in rat-liver mitochondria by Ernster & Navazio (1957) suggested that a study of the oxidation of *iso*citrate in ox-adrenocortical mitochondria might help to explain the relatively feeble steroid 11β-hydroxylation in the presence of this tricarboxylic acid.

A preliminary account of some of the experiments described here has been published (Grant & Mongkolkul, 1958).

EXPERIMENTAL

Enzyme preparations, materials and methods

Suspensions of mitochondria were prepared from finely minced, fresh ox-adrenocortical tissue as described by Brownie & Grant (1954). TPN^+ (about 95% pure) and crystalline adenosine triphosphate were purchased from Sigma Chemical Co. (U.S.A.); TPN^+ was assayed by the glucose 6-phosphate dehydrogenase method of Horecker & Smyrniotis (1955); DPN^+ (about 95% pure) and DPNH were purchased from Boehringer and Sons, Mannheim-Waldhof, Germany, and their purities checked by alcohol dehydrogenase from the same firm. TPNH was prepared by the method of Conn, Kraemer, Pei-Nan & Vennesland (1952). DL-*iso*Citric lactone was purchased from L. Light and Co., and converted into *iso*citrate by the method of Krebs & Eggleston (1944). Cytochrome *c* was purchased from L. Light and Co. and assayed by the method of Potter (1949). 2:6-Dichlorophenolindophenol was purchased from British Drug Houses Ltd.

Mitochondria, suspended in 0·25 M-sucrose containing 0·05 M-potassium phosphate, pH 7·4, were depleted of native pyridine nucleotides by pre-incubation, termed 'aging', at 37°, with gentle shaking (Huennekens & Green, 1950). Mitochondria from about 1 g. of adrenal cortex were contained in 1 ml. of suspension.

Oxygen-consumption measurements were made by the usual Warburg technique.

Changes in optical density were observed by using a Unicam SP. 500 spectrophotometer with a water-jacketed cuvette holder for the maintenance of constant temperatures.

For the determination of the pyridine nucleotide specificity of the mitochondrial *iso*citric dehydrogenase the cytochrome system was blocked with cyanide and 2:6-dichlorophenolindophenol was used as a terminal hydrogen acceptor according to the method of Ernster & Navazio (1957). The reaction was initiated by the addition of *iso*citrate, and readings were taken at 600 mμ at intervals against a blank containing all additions except pyridine nucleotides.

For the determination of activities of reduced pyridine nucleotide oxidases and pyridine nucleotide transhydrogenase cytochrome *c* was employed as electron acceptor and observations were made at 340 mμ with occasional stirring of the cuvette contents for aeration.

RESULTS

Comparison of rates of oxidation of citrate, iso*citrate and fumarate by ox-adrenocortical mitochondria*

The results, illustrated in Fig. 1, show that citrate and DL-*iso*citrate are readily oxidized by ox-adrenocortical mitochondria at rates which are not significantly different from that observed with fumarate as substrate.

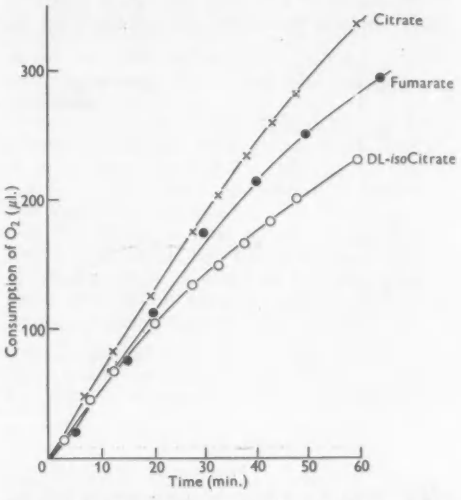

Fig. 1. Oxidation of citrate, DL-*iso*citrate and fumarate by mitochondria from ox-adrenal cortex. Each Warburg flask contained, in 3 ml. final volume, 0·5 ml. of a suspension in 0·25 M-sucrose of mitochondria, which had not been aged, from 1 g. of adrenal cortex, 150 μmoles of potassium chloride, 12 μmoles of magnesium sulphate, 120 μmoles of potassium phosphate buffer, pH 7·4, 3 μmoles of adenosine triphosphate and 30 μmoles of potassium citrate or potassium fumarate or 60 μmoles of potassium DL-*iso*citrate. The gas phase was air, and the flasks were shaken in a water bath at 37°. The tricarboxylic acids and fumarate were tipped in at zero time.

Pyridine nucleotide specificity of isocitric *dehydrogenase in ox-adrenocortical mitochondria*

Fig. 2 shows the effects of added pyridine nucleotides on the oxidation of *iso*citrate by aged mitochondria from ox-adrenal cortex. These results suggest that oxidation of *iso*citrate in the mitochondria proceeds mainly by way of a DPN^+-specific *iso*citric dehydrogenase. The possibility, however, remains that the oxidation involves a TPN^+-linked enzyme working with traces of TPN^+ remaining in the aged mitochondria, and that the observed DPN^+ reduction results from the activity of a pyridine nucleotide transhydrogenase according to the reaction

$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH$$

suggested by Kaplan, Swartz, Frech & Ciotti (1956). This possibility was investigated in the following experiments.

Activity of reduced pyridine nucleotide oxidases of pyridine nucleotide transhydrogenase in ox-adrenocortical mitochondria

The results of a typical experiment shown in Fig. 3 indicate that aged mitochondria from ox-adrenal cortex contain an active DPNH oxidase, that TPNH is not oxidized at an appreciable rate in this system and that there is no significant reduction of added DPN^+ by TPNH. In a similar experiment added TPN^+ was found to be without significant effect on the rate of oxidation of DPNH. It may therefore be concluded that under these experimental conditions the activity of pyridine nucleotide transhydrogenase in these mitochondria is negligible and that it cannot account for the reduction of DPN^+ observed on addition of *iso*citrate in the experiments described above.

DISCUSSION

From the results described here it is obvious that mitochondria from ox-adrenal cortex can oxidize citrate and *iso*citrate at approximately the same rate as fumarate. This is in agreement with the observations of Sourkes & Heneage (1952) on whole homogenates of ox-adrenal glands. Rates of oxidation by mitochondria, of the same order and under similar conditions, are observed with succinate as substrate (Brownie, Grant & Davidson, 1954). Thus permeability barriers to the entry of

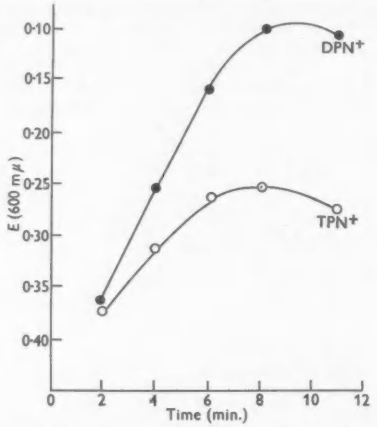

Fig. 2. Effect of added pyridine nucleotides on the oxidation of *iso*citrate by mitochondria from ox-adrenal cortex. The mitochondria were aged for 1·5 hr. at 37° as described in the Experimental section. Each cuvette of 5 mm. light path contained mitochondria from about 50 mg. of adrenal cortex, 1 μmole of manganese chloride, 10 μmoles of magnesium sulphate, 120 μmoles of potassium phosphate buffer, pH 7·4, 3 μmoles of potassium cyanide, 0·5 μmole of DPN^+ or TPN^+, as indicated, 0·19 μmole of 1:6-dichlorophenolindophenol and 20 μmoles of DL-potassium *iso*citrate in a final volume of 1·5 ml. The final pH was 7·4 and the temperature 20°. The blank cuvette contained all substances other than pyridine nucleotide. The *iso*citrate was added at zero time.

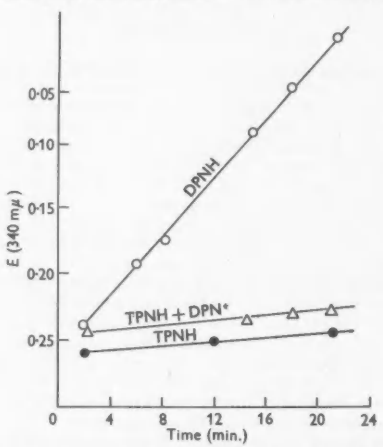

Fig. 3. Oxidation of reduced pyridine nucleotides and the activity of pyridine nucleotide transhydrogenase in mitochondria from ox-adrenal cortex. The mitochondria were aged for 1·5 hr. at 37°. Each cuvette of 5 mm. light path contained mitochondria from about 50 mg. of adrenal cortex, 60 μmoles of potassium phosphate buffer, pH 7·4, 0·02 μmole of cytochrome *c*, 0·25 μmole of DPNH, TPNH and DPN^+, as indicated, in a final volume of 1·5 ml. The final pH was 7·4 and the temperature 20°. The blank cuvette contained all substances other than pyridine nucleotides, which were added to the other cuvettes at zero time. Aeration was maintained by occasional stirring with glass rods.

citrate into the mitochondria (Johnson & Lardy, 1958) or the deficiency of these particles in aconitase (Dickman & Speyer, 1954) are unlikely to be limiting factors which might account for the relatively feeble 'stimulation' of 11-deoxycorticosterone 11β-hydroxylation by citrate as compared with fumarate (Grant & Brownie, 1955; Sweat & Lipscomb, 1955). The results now obtained suggest that *iso*citrate oxidation in ox-adrenocortical mitochondria is linked predominantly with the reduction of DPN^+ rather than TPN^+. Since TPNH is required for steroid hydroxylation this would explain the relatively feeble effect of citrate. The possibility has not been excluded that a selective destruction of a TPN-specific *iso*citric dehydrogenase occurs during ageing at 37°, but this is not thought to be likely since under some circumstances the TPN^+-linked enzyme from pig heart may be heated to 56–57° for 15 min. with only slight loss of activity (Hogeboom & Schneider, 1950) and the thermal lability of the DPN^+-linked enzyme may be used as a means of freeing a yeast TPN^+-*iso*citric dehydrogenase of this activity (Kornberg, 1955).

The feeble oxidation of TPNH by the mitochondrial flavoproteins and cytochrome system as compared with that of DPNH is in keeping with the view first expressed by Colowick (1951) that TPNH oxidation is not normally coupled with phosphorylation and with numerous observations of biosynthetic reactions which require TPNH. Examples of such reactions are steroid hydroxylations (Grant, 1956*b*), the concerted cyclization and hydroxylation of squalene in sterol formation (Clayton & Bloch, 1956), the reversal of fatty acyl-coenzyme A dehydrogenase activity in fatty acid synthesis (Lynen & Decker, 1957), the synthesis of fructose in male reproductive organs (Hers, 1956) and the incorporation of C_1 units into serine (Hatefi, Osborn, Kay & Huennekens, 1957). These observations suggest a special role for TPNH as a specific reductant in biosynthetic reactions rather than as a substrate for oxidations via flavoproteins and cytochromes coupled with phosphorylations. In the adrenal cortex the necessity of conserving TPNH for steroid-hormone biosynthesis is obvious.

Oxidation of TPNH in the adrenal cortex via DPN^+ with the aid of a pyridine nucleotide transhydrogenase offers the possibility of controlling the TPNH level and through it steroid biosynthesis. Transhydrogenase activity was not, however, demonstrated under the present experimental conditions, which were designed to investigate the pyridine nucleotide specificity of the mitochondrial *iso*citric dehydrogenase. Sweat & Lipscomb (1955), however, demonstrated transhydrogenase activity in ox-adrenocortical mitochondria indirectly by their observations that 11β-hydroxylation of 11-deoxycorticosterone by these mitochondria is negligible in the presence of TPN^+ or DPNH alone, but that hydroxylation occurs equally well in the presence of either TPNH or DPNH along with small amounts of TPN^+. Failure to observe transhydrogenase activity in the present investigation may be associated with the ageing of the mitochondria, and it is tempting to speculate that this ageing may result in loss of minute traces of 17-hydroxysteroids, notably oestradiol-17β, which according to Talalay & Williams-Ashman (1958) may function as co-enzymes for pyridine nucleotide transhydrogenase. This possibility is at present under investigation.

SUMMARY

1. The feeble 'stimulation' of 11-deoxycorticosterone 11β-hydroxylation in mitochondria from ox-adrenal cortex by citrate is not due to failure of this substance to enter the mitochondria or to be oxidized via *iso*citrate, but may be related to the fact that *iso*citrate oxidation which appears to be predominantly diphosphopyridine nucleotide linked in these mitochondria fails to produce adequate amounts of reduced triphosphopyridine nucleotide.

2. The reduction of diphosphopyridine nucleotide by the mitochondrial enzymes in the presence of *iso*citrate, under the conditions described, cannot be attributed to the activity of a pyridine nucleotide transhydrogenase.

3. The failure to oxidize reduced triphosphopyridine nucleotide by flavoprotein–cytochrome systems in the mitochondria and the possible significance of the transhydrogenase reaction in relation to biosynthetic reactions are discussed.

We are indebted to Mr R. Bayne for skilled technical assistance and to the Medical Research Council for a grant from which the expenses of the work were partly defrayed.

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The Effect of Haemorrhage on the Incorporation of [α-^{14}C]Glycine into Stercobilin

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It was shown by Tarchanoff (1874) that injected haemoglobin gave rise to bile pigments. This observation led eventually to an approximate correlation of the rate of haemoglobin breakdown with the daily amounts of bile pigment produced (Eppinger & Ranzi, 1920; Hawkins, Shribhishaj, Robscheit-Robbins & Whipple, 1931; Miller, Robscheit-Robbins & Whipple, 1945). From the nature and arrangement of the β-substituents of biliverdin, from which the other naturally occurring bile pigments are derived, it is evident that, of the free porphyrins, only protoporphyrin IX could theoretically give rise directly to biliverdin. Evidence for such a conversion has been obtained by using isotopically labelled protoporphyrin (London, Yamasaki & Sabella, 1951); however, when a rabbit's liver was perfused with a solution of protoporphyrin only 2% of the porphyrin was recovered, while the remainder did not give rise to bile pigments (Watson, Pass & Schwartz, 1941; Salzburg & Watson, 1941). Since it is known that bile pigments are formed in the liver, it may be inferred that they arise directly only from haem (London, 1950) or from haem proteins.

Isotope studies have shown that bile pigment is derived not only from red cells at the end of their life span, but also from a second source, responsible for the labelling of faecal stercobilin during the first few days after feeding isotopically labelled glycine. The origin of this second fraction of bile pigments, indistinguishable chemically from the catabolic fraction produced from the breakdown of red cells at the end of their life span, is unknown but the increase in percentage of this fraction from about 10% in the normal person to about 80 or 90% of the bile pigments in patients with congenital porphyria, pernicious anaemia and sickle-cell anaemia, conditions associated with increased haemopoiesis, has suggested that this fraction may arise in association with haemopoiesis. Since this suggestion has been confirmed by our findings described in the present paper, the early labelled fraction may conveniently be called the haemopoietic fraction. It has been suggested that the haemopoietic fraction may arise in the bone marrow from premature breakdown of haemoglobin from intact red cells (London, Shemin, West & Rittenberg, 1949; Gray, Neuberger & Sneath, 1950), or from haem in excess of globin (Neuberger, Muir & Gray, 1950). On the other hand, direct synthesis from pyrrolic compounds without the formation of haemoglobin as an intermediate has been considered (London, West, Shemin & Rittenberg, 1950), and some support for this was obtained by Berlin, Neuberger & Scott (1956), who showed that a proportion of δ-aminolaevulic acid admin-

istered to man is rapidly converted into stercobilin by a pathway which may not include haemoglobin. If the formation of the haemopoietic fraction of the bile pigment is related to haemopoiesis, there should be proportionality between the haemopoietic activity in the marrow and the labelling of stercobilin during the first few days after feeding isotopic glycine. If, on the other hand, this fraction arises, for example, from the turnover of haem-containing enzymes in the liver or in the body as a whole (Theorell, Beznak, Bonnichsen, Paul & Åkeson, 1951), then its rate of production should not be affected by changes in the rate of blood formation. Haemopoiesis may be stimulated by bleeding, and it seemed that this problem might be studied by examining the incorporation of [α-^{14}C]-glycine into the faecal stercobilin before and after venesection. The patient who was the subject for this study was suffering from a form of porphyria characterized by mild intermittent photosensitivity (porphyria cutanea tarda) in contrast with the continuous severe sensitivity associated with congenital porphyria. There is evidence that the former type of porphyria is not related to a defect specifically in the erythropoietic system (Watson, Lowry, Schmid, Hawkinson & Schwartz, 1951), and it was shown by Lowry, Hawkinson & Watson (1952) that the formation of bile pigment in porphyria cutanea tarda is normal, 85–90% occurring in the catabolic fraction. A preliminary report of the present work has been published (Gray & Scott, 1958).

EXPERIMENTAL

Clinical material

The subject was a man of 53 years with very mild porphyria cutanea tarda. He was in excellent general health. Blisters on the backs of the hands, and on the forehead and cheeks, which had developed on exposure to sunshine a year before, were almost completely healed. No lesions were present on the covered parts of the body and when sunshine was avoided the lesions healed with minor scarring. There was no excessive pigmentation or hirsutism, nor abdominal or neurological symptoms suggestive of acute porphyria. There was no anaemia, and no abnormality of mean corpuscular haemoglobin or of the differential white-cell count except for a slight eosinophilia of 8%. The blood film was normal. The detailed results of other haematological examinations are presented below. Consumption of alcohol had not been excessive and on general examination there was no abnormality except for a blood pressure of 200/100 mm. Hg (systolic/diastolic). There was no history of jaundice, no evidence of abnormal liver function, and at the time of investigation the urine did not contain an excess of porphyrins; porphobilinogen had never been detected in the urine. The faeces contained a small but significant excess of coproporphyrin. During the 34 days of the investigation the mean quantities of faecal coproporphyrin and protoporphyrin excreted were 1·8 ± 1·1 mg. (0·84–4·81) and 0·5 ± 0·23 mg. (0·32–1·08)/day respectively.

Methods

Isolation of faecal stercobilin. The faeces were ground in a mortar with about a quarter of their bulk of acetic acid and then with an equal volume of ether. After standing, a deeply pigmented layer separated and was decanted through filter paper. After repeated extraction of the residue with further quantities of ether, acetic acid being added at about every fourth extraction, the united extracts were distilled on the water bath and the resulting dark brown solution, containing much acetic acid, was added to ten times its volume of 0·27 N-HCl. After it had stood overnight the dark-brown fatty material was filtered off through a large Büchner funnel and saturated potassium acetate solution added to the filtrate until it was no longer acid to Congo red. This solution was repeatedly extracted with ether until the ether extract showed only weak porphyrin absorption bands with a hand spectroscope. The ethereal extract was analysed for porphyrins and the aqueous phase was extracted with chloroform. The stercobilin hydrochloride was then isolated as previously described (Gray *et al.* 1950).

Estimation of faecal porphyrins. The united ethereal extracts were extracted with 0·15 N-HCl until the acid extract showed no significant absorption bands in the hand spectroscope. The volume was noted and after suitable dilution the coproporphyrin was estimated spectrophotometrically at 401 mμ ($E^{1\%}_{1cm.}$ 6500). The remaining ethereal solution was exhaustively extracted with 0·7 N-HCl and the protoporphyrin determined spectrophotometrically at 409 mμ ($E^{1\%}_{1cm.}$ 5000). The optical densities were not corrected for interfering substances.

Blood volume. This was determined with ^{51}Cr by a method modified from that of Mollison & Veall (1955).

Blood haemoglobin. This was determined by the method of Haldane (1900), by using the British Standards Institution standard.

Measurement of radioactivity. All samples of stercobilin were kindly gas-counted by Dr A. S. McFarlane with the method of Bradley, Holloway & McFarlane (1954); the standard error was less than 3%.

Faecal urobilinogen. This was measured by the method of Schwartz, Sborov & Watson (1944) as modified by Gray (1957).

Plan of experiment

The time relationship between the various phases of the experiment is shown in Fig. 1, together with relevant haematological data. [α-^{14}C]Glycine (25 μc, 4·957 mg.) was dissolved in 50 ml. of water and drunk by the patient, the vessel being washed out with water (6 × 20 ml.). The faeces were subsequently collected during the succeeding 17 days at intervals of 2, 2, 3, 6 and 4 days. On the 15th day of the experiment, two 540 ml. quantities of blood were removed by two venesections separated by about 3 hr. Haematological examinations were carried out at convenient intervals throughout the investigation. On the 17th day of the experiment the reticulocyte count had risen from 0·2 to 0·6%, showing that haemopoiesis had been stimulated, and a second dose of 25 μc of [α-^{14}C]glycine was given, precisely as before. The reticulocyte count continued to increase up to the twenty-third day. Faecal collections were continued

and worked up at the same intervals as in the preliminary experiment. Stercobilin hydrochloride was isolated from each batch of faeces as soon as collection was complete. Stercobilin was also isolated from faeces collected at convenient intervals over the period 130–157 days after the first administration of glycine, that is 113–140 days after the second administration. It was not possible to collect samples of faeces over the period 110–130 days, when the red cells formed during the first administration of labelled glycine were being broken down.

RESULTS AND DISCUSSION

It is convenient to consider together the quantities and radioactivities of the stercobilin obtained at corresponding intervals in phases I and II, the 17-day periods before and after the second dose of labelled glycine.

The rates of excretion of stercobilin during the two 17-day periods are shown in Table 1. Fig. 1 shows the haematological data obtained during the experiments. The concentration of haemoglobin fell from 17·5 to 14·5 g./100 ml. after the bleeding and was 14·2 g./100 ml. at the time of the first collection of faeces, and had risen to 16·3 g./100 ml. 3 days before the end of the experiment. As is usual in values for excretion of bile pigment determined in this way (see, for example, Gray & Neuberger, 1952) there was considerable variation in the rates of excretion even during phase I when the total haemoglobin was not changing. It may be seen from Table 1 and Fig. 1, however, that after the removal of blood there was a substantial increase in reticulocyte count and an apparent decrease of over 40% in the average rate of excretion of stercobilin. This apparent decrease is considered to be too great, for reasons which are discussed below.

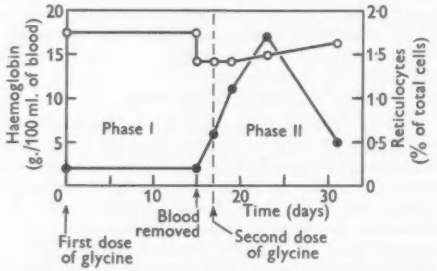

Fig. 1. Plan of experiment and relevant haematological data. On both occasions the [α-^{14}C]glycine given contained 25 μc in 4·957 mg. The volume of blood removed was 1·08 l.; the total volume of blood was 5·5 l. throughout phases I and II, both of which lasted for 17 days. ○, Concentration of haemoglobin (left-hand scale of ordinates); ●, reticulocyte count (right-hand scale of ordinates).

The observed specific radioactivities of the stercobilin isolated from the faeces during each interval are shown in Fig. 2. The radioactivities in phase II are considerably higher than in phase I, but the values for the specific activities during

Table 1. *Excretion of stercobilin before and after removal of blood*

The time intervals are measured from the day when isotopic glycine was first given. After 17 days a second dose of glycine was given; the faeces were then collected at intervals corresponding to those in the first period. Blood (1·08 l.) was removed on the 15th day. The method for estimating stercobilin gives values which include stercobilinogen.

Collection period (days)	No. of days	Wt. of faeces (g.)	Stercobilin excreted (mg./day)
Phase I			
1–2	2	140	111
3–4	2	406	156
5–7	3	250	87
8–13	6	440	141
14–17	4	705	120
		Mean	125
Phase II			
18–19	2	350	79
20–21	2	220	59
22–24	3	474	92
25–30	6	823	55
31–34	4	769	87
		Mean	72

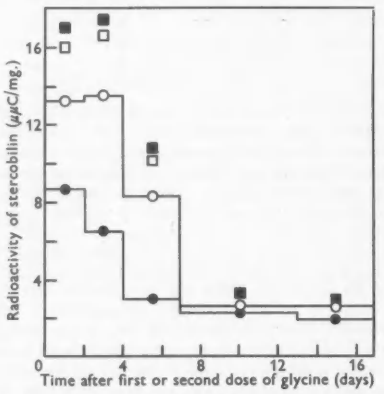

Fig. 2. Mean specific activities of stercobilin after feedings of [α-^{14}C]glycine before and after removal of blood. ●, During phase I; ■, during phase II; □, phase II, corrected for residual radioactivity from phase I; ○, corrected both for residual radioactivity and for changes in the total quantity of circulating haemoglobin during phase II. For further details of corrections, see text and Fig. 1.

phase II require correction because a proportion of the stercobilin would still have been labelled as a result of the [α-^{14}C]glycine given at the start of phase I. When the specific activity of the stercobilin during phase I is plotted on a logarithmic scale against time, an approximately linear relationship is obtained, corresponding with a half-life of the faecal haemopoietic stercobilin of about 6 days. It was therefore assumed that the approximately exponential decrease of radioactivity of the haemopoietic fraction during phase I was maintained with respect to the first dose of glycine throughout phase II, and the small extrapolated values were subtracted from the specific activities of the haemopoietic fraction in phase II. This has permitted the correction of the observed specific activities of the stercobilin isolated after the second feeding of glycine for the persistence of labelling resulting from the first administration. A second correction is necessary to permit a valid comparison of the specific activities of the stercobilin samples because, after the bleeding, the circulating haemoglobin was decreased and caused a decrease in the quantity of unlabelled catabolic stercobilin derived from red cells at the end of their life span. This would increase the specific activity of the combined fractions whether any increased incorporation into the early fraction took place or not. The values, corrected for residual radioactivity, were accordingly multiplied by the ratio of the mean haemoglobin concentration in the appropriate interval in phase II to the haemoglobin concentration in phase I; the resulting values give approximately the specific activities which would have been observed had there been no change in the total haemoglobin. These corrections, particularly the second, lower the values in phase II by about 20 %, but the values are still substantially higher throughout than those in the first period.

Table 2 shows the ratios of the corrected specific activities of stercobilin isolated at corresponding intervals in phases I and II. The ratio was always greater than unity and reached a value of 2·89 between the 5th and 8th days after the glycine feeding.

Table 2. *Incorporation of* [α-^{14}C]*glycine into faecal stercobilin before and after haemorrhage*

The time intervals relate to corresponding intervals after giving isotopic glycine before (phase I) and after (phase II) haemorrhage. The corrected values of the specific activities have been used in calculating the ratios; for further details see text.

Days after glycine feeding	Specific activities of faecal stercobilin (ratio: phase II/phase I)
1–2	1·48
3–4	2·07
5–7	2·89
8–13	1·00
14–17	1·35

It is possible to calculate the output of ^{14}C-labelled stercobilin from the results of the estimations shown in Table 1. Such a calculation is, however, rendered invalid because of the variation in the daily weight of faeces (see Table 1) and of the unpredictable destruction of a part of the bile pigment in the gut to compounds not reacting with Ehrlich's aldehyde reagent under the conditions of the estimation; moreover, this destruction may be affected by diet. A further consequence of such destruction is that relatively more may be lost when less is present.

It is therefore more valid to assume a daily production of catabolic bile pigment approximately equivalent to 1/120 of the total haemoglobin in the body; since there is no reason to suppose that labelled material will be destroyed at a different rate in the gut from unlabelled material, the total amounts of stercobilin ^{14}C produced can be determined by numerical integration of the two curves of specific activity plotted against time. The total radioactivities calculated in this way amount to 62·4 and 104·4 $\mu\mu$c before and after the bleeding respectively; there was thus an increase of about 60 % in the incorporation of glycine into the haemopoietic stercobilin.

Blood was removed towards the end of the first period and the second dose of labelled glycine was given at a time when the response of the bone marrow to the blood loss was established. This was judged by reticulocyte counts of the circulating blood and it was reckoned that the peak of activity in the bone marrow would precede the maximum reticulocyte count by three or four days. Reference to Fig. 1 shows that the glycine was probably given a few days too early to achieve a maximum effect. Moreover, the methods of correction used, to permit a direct comparison of the specific activities of the stercobilin in the two periods, are only approximate. In particular, correction for decreased dilution by catabolic pigment in the second period has been based on the approximation that all the stercobilin arises from haemoglobin breakdown whereas in fact only 85–90 % is normally so derived. Nevertheless, it is considered that the increases in specific activity are considerably greater than could arise from errors in the corrections. It is therefore concluded that the haemopoietic stercobilin is at least in part correlated with the biosynthesis of haemoglobin. We have not sufficient data to decide whether other sources of haemopoietic stercobilin exist or not.

Table 3 shows the specific activity of the faecal stercobilin hydrochloride isolated between the 129th and 157th day of the experiment, i.e. at a

Table 3. *Radioactivity of faecal stercobilin when labelled erythrocytes were being broken down*

The time intervals are measured from the day when isotopic glycine was first given.

Collection period (days)	Wt. of faeces (g.)	Specific activity of stercobilin hydrochloride ($\mu\mu$C/mg.)
130–131	540	12·0
132–135	550	14·4
136–145	1160	10·9
146–149	510	13·5
150–153	350	9·3
154–157	500	9·0

time when the red cells labelled after the second glycine feeding were breaking down. Unfortunately it was not possible to collect faeces at the time when red cells labelled from the first glycine experiment were being broken down.

In patients with congenital porphyria, the total daily excretion of stercobilin is greatly increased. Further, the studies with isotopic glycine (Gray *et al.* 1950; London *et al.* 1949) have shown that 80–90% of the isotope is excreted in the haemopoietic fraction; this has been interpreted to indicate that the increased excretion of bile pigment is due to an increase in the actual quantity of the haemopoietic fraction. It is of interest, therefore, that at a time at which the red cells labelled after the second feeding of [*a*-^{14}C]glycine would be expected to be breaking down maximally, the specific activity of the catabolic stercobilin was showing a broad peak at about 14 $\mu\mu$C/mg., a value which is slightly higher than the corrected maximum specific activity corresponding with the much narrower peak of the early fraction (13·5 $\mu\mu$C/mg.) measured during phase II (cf. Gray *et al.* 1950). Although these figures cannot be corrected for the persistence of labelling from the breakdown of red cells labelled during the first feeding of [α-^{14}C]glycine they show clearly that the labelling of the stercobilin at this time was as great as that observed during the first few days, and is not made extremely low by dilution with large amounts of haemopoietic stercobilin. Porphyria cutanea tarda thus differs from the congenital form of the disease in this respect, in confirmation of the earlier findings of Lowry *et al.* (1952).

SUMMARY

1. The incorporation of [α-^{14}C]glycine into the early-labelled ('haemopoietic') stercobilin has been studied in man before and after haemopoiesis had been stimulated by bleeding.

2. The radioactivity of stercobilin formed after haemorrhage was consistently greater than the radioactivity of that formed in the corresponding period before haemorrhage, showing that the haemopoietic stercobilin is related to the rate of production of red cells.

3. The subject studied was suffering from a mild form of porphyria cutanea tarda. A comparison of the specific activities of stercobilin excreted between days 1 and 34, and 129 and 157 days after the first feeding of glycine, shows that there was no increase in the amount of haemopoietic stercobilin relative to late-labelled stercobilin such as occurs in congenital porphyria, in confirmation of the findings of other workers.

We are grateful to the Medical Research Council for an expenses grant to one of us (C. H. G.), to Dr D. C. Nicholson, Miss Thelma Beck and Miss Mary Pickett for isolation of the stercobilin and to Dr A. S. McFarlane of the National Institute for Medical Research for estimating the radioactivity of the specimens. We also thank Dr R. D. Sweat for allowing the patient to come to King's College Hospital for investigation and Dr D. I. Williams for making available a bed.

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The Isolation of a Sixth Kober Chromogen from the Urine of Pregnant Women and its Identification as 18-Hydroxyoestrone

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In the course of column partition-chromatographic analysis of ketonic–phenolic fractions obtained from ether extracts of enzymically hydrolysed pregnancy urine, Kober-chromogenic material slightly more 'polar' in its chromatographic behaviour than 16α-hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16α-diol) was regularly detected. The two possibilities were at first considered that this material might have arisen by 'leakage' of the chromatographically similar 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) into the ketonic fractions or by 'tailing' of the 16α-hydroxyoestrone fraction on the columns. However, when a concentrate of this Kober-chromogenic material was subjected to a second Girard separation and the resulting ketonic fraction rechromatographed in the same system nearly 60% of the starting material was recovered. This finding disposed of these two possibilities and it became evident that a hitherto unknown ketonic Kober chromogen (KC-6) had been detected.

Subsequently it was found that 'KC-6' was in fact a mixture of two different Kober chromogens; and in the present paper the isolation and complete characterization of one of these (KC-6A) is described. Brief preliminary accounts of the main findings reported here have been published (Loke, Watson & Marrian, 1957; Loke, Marrian, Johnson, Meyer & Cameron, 1958).

EXPERIMENTAL AND RESULTS

Methods

Hydrolysis and extraction of urine. The procedure used for preparing ketonic–phenolic fractions from enzymically hydrolysed urine was essentially similar to that described by Marrian, Watson & Panattoni (1957), but two modifications of this procedure were introduced: (i) as the result of a suggestion made to us by Dr J. R. K. Preedy (The London Hospital) Bradosol (5% solution of β-phenoxyethyldimethyldodecyl ammonium bromide; Ciba Laboratories Ltd.) was added to the hydrolysed urine (1 ml. of Bradosol/l. of urine) immediately before the initial extraction with ether. This almost completely eliminated the formation of troublesome emulsions. (ii) To ensure that the ketonic–phenolic fractions were not contaminated with non-ketonic material the Girard separation with trimethylammoniumhydrazide chloride was carried out twice in succession.

Column-partition chromatography. Large-scale partition chromatograms on Celite columns with the solvent system methanol–water–hexane–benzene (70:30:20:80, by vol.) were carried out in a constant-temperature room (25°) as described by Marrian *et al.* (1957). The ketonic–phenolic concentrate from about 200 l. of urine was applied to each column and the eluate collected in 40 ml. fractions. Fractions 30–45, containing KC-6, were combined and evaporated to dryness. Since such KC-6 concentrates were usually contaminated with the 'tailings' of the KC-5 fraction (Marrian *et al.* 1957) they were always rechromatographed after combining into batches corresponding to 400–450 l. of urine.

Paper chromatography. Two solvent systems of the Bush (1952) type were employed throughout this work: system 4, methanol–water–chloroform–benzene (66:33:36:64, by vol.); system 8, methanol–water–hexane–benzene (70:30:50:50, by vol.). Whatman no. 42 papers, previously extracted exhaustively in a Soxhlet apparatus with a mixture of methanol, chloroform and benzene, were used.

Amounts of urinary concentrates or of pure substances containing 5–50 μg. of Kober chromogen (calculated as oestriol) were applied in methanolic solution to the papers, which were then equilibrated in the tanks overnight at 18° and developed for about 6 hr. After drying in air the papers were sprayed with diluted (1 in 5) Folin–Ciocalteu reagent and the spots developed by exposure to ammonia vapour in a sealed tank.

Under the conditions employed rather variable R_F values were obtained with pure oestrogens, and accordingly standard substances were always run on the same papers as the unknowns. For system 4 the standards used were 17-*epi*oestriol (R_F about 0·50) and oestriol (R_F about 0·15), and for system 8oestrone (R_F about 0·50) was the standard.

Miscellaneous. The Kober reaction was carried out by the method of Brown (1952), as modified by Bauld (1954), with the 'oestriol reagent' described by the latter.

Melting points are uncorrected for emergent stem.

Evidence for the presence of two different Kober chromogens in KC-6

Preliminary experiments on the purification of KC-6 were carried out on 26 mg. of a chromatographically prepared concentrate obtained as a brown viscous oil from 400 l. of late-pregnancy urine. Paper chromatography with system 4 showed an intense well-defined spot with R_F about 0·50, and, since only faint spots appeared elsewhere on the chromatogram, it was assumed at first that the material might be nearly homogeneous. However, after reduction of a portion of

the original concentrate with sodium borohydride in methanolic solution and treatment of the product with Girard's reagent T (trimethylammonium-hydrazide chloride; Girard & Sandulesco, 1936) in the usual way to remove any unreduced ketonic material paper chromatography in the same system showed the presence of two main spots of about equal intensities. One of these had the same R_F as that of the unreduced material, and the other had R_F about 0·15.

From the main bulk of the original KC-6 concentrate a small amount of crystalline material was obtained by rapid leaching with chilled acetone. The acetone-soluble solid on treatment with 0·1 ml. of chloroform at −20° yielded a somewhat larger amount of crystalline material. The two crystalline fractions were combined and purified by leaching again with chloroform at −20° and finally recrystallizing from methanol–benzene. The nearly white crystalline product (6 mg.) had m.p. 247–250° (sealed evacuated capillary). In the Kober reaction it gave a colour, the optical density of which at 512·5 mμ was only 19% of that of the colour given by the same weight of oestriol. On paper chromatography (system 4) a single spot was detected which had the same R_F (about 0·50) as that of the main component in the original KC-6 concentrate.

A portion of the crystalline material was reduced with sodium borohydride in methanolic solution. On paper chromatography (system 4) the reduction product showed an intense spot with the same R_F as that of the unreduced material and a second faint spot with R_F about 0·15. In the Kober reaction the intensity of the colour given by the reduction product was about 200% greater than that of the colour given by the unreduced substance.

The amorphous fraction recovered from the original KC-6 concentrate soluble in acetone and chloroform gave a positive Kober reaction and, like the original concentrate, showed a main spot with R_F about 0·50 on paper chromatography with system 4. However, in marked contrast with the behaviour of the crystalline fraction, reduction by sodium borohydride of the amorphous fraction yielded a product which gave a Kober reaction of virtually the same intensity as that given by the unreduced material. Furthermore, on paper chromatography (system 4), the reduction product showed an intense spot with R_F about 0·15 and a much less intense spot with R_F 0·50.

On the basis of these findings it was concluded that the original KC-6 concentrate must have contained two different Kober chromogens which could not be distinguished from one another by paper chromatography in the solvent system used but which could be partially separated by leaching with acetone and chloroform. It seemed evident that one of these, which was provisionally designated as KC-6A, can be reduced with sodium borohydride to form a product which has the same R_F in the solvent system used as the parent compound, but which is a much stronger Kober chromogen than the latter: the other, provisionally designated as KC-6B, yields a reduction product which is considerably more 'polar' in its chromatographic behaviour than the parent compound, but which is not a stronger Kober chromogen than the latter.

The evidence obtained indicates that the purified crystalline fraction must have been a nearly pure specimen of KC-6A contaminated with no more than a trace of KC-6B; the amorphous fraction which was more soluble in acetone and chloroform must have contained predominantly KC-6B, but also an appreciable amount of KC-6A.

Properties of KC-6A: provisional identification as 18-*hydroxyoestrone*

General. A KC-6 concentrate weighing 84 mg. was prepared from 504 l. of late-pregnancy urine. By leaching this twice in succession at −20° with 0·2 ml. volumes of chloroform a nearly white solid was obtained. After crystallization from methanol–benzene the product (11·8 mg.) was combined with 4 mg. of the material remaining from the previous batch (see above). The combined material was leached once with 0·2 ml. of chloroform at −20° and recrystallized once from methanol–benzene and once from ethanol. The final product was obtained in a yield of 5·7 mg. and had m.p. 255–257° (sealed evacuated capillary), $[\alpha]_D^{19} + 146°$ (*c*, 0·369 in ethanol), and ϵ_{281} 2200 in ethanol (Found: C, 75·4; H, 7·9. Calc. for $C_{18}H_{22}O_3$: C, 75·5; H, 7·7%).

The KC-6A recovered from the optical-rotation determination was combined with 2·3 mg. of material recovered from the mother liquors of the analytical sample and acetylated in the usual way. The product was recrystallized twice from ethanol, yielding a product which had m.p. 162–165° (hot stage) (Found: C, 70·8; H, 7·0. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%).

In the Kober reaction the preparation of KC-6A gave a colour the intensity of which ($E_{512\cdot5m\mu}$) was 21% of that of the colour given by the same weight of oestriol. The intensity of the Kober colour given by the sodium borohydride reduction product of the preparation was 133% greater than that of the colour given by the unreduced substance. The absorption spectra of the Kober colours of KC-6A and of its reduction product are shown in Fig. 1. It will be seen that whereas the spectrum of the colour given by the reduction product is normal with a single maximum at 512·5 mμ, that of the colour given by KC-6A itself shows a second maximum at 420 mμ of about the same intensity as that at 512·5 mμ.

The absorption spectra of solutions of KC-6A in conc. H_2SO_4 and of its sodium borohydride-reduction product after standing for 2 hr. at 24° are shown in Fig. 2. In the 'blue tetrazolium' test as carried out by Mader & Buck (1952) KC-6A showed negligible reducing power.

Since KC-6A is ketonic and has the probable formula $C_{18}H_{22}O_3$ and since it gave a positive Kober reaction the working hypothesis was adopted that it might be a 17-hydroxy or 17-oxo derivative of oestra-1:3:5-trien-3-ol with an oxo or hydroxy substituent elsewhere in the molecule. As KC-6A showed negligible reducing power towards 'blue tetrazolium' C-16 could be excluded as the other substituted position.

Since KC-6A and its sodium borohydride-reduction product had the same R_F in the solvent system used it seemed possible that in the latter compound the two alcoholic hydroxyl groups might be influencing one another by steric hindrance or by hydrogen bonding. Substitution at C-16 had been excluded and the possibility of substitution at C-18 was therefore considered.

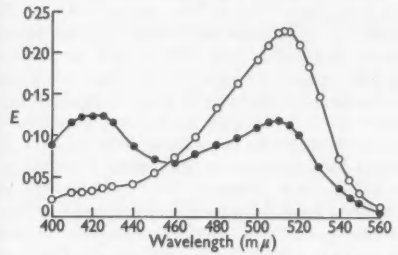

Fig. 1. Absorption spectra of colours given in the Kober reaction by KC-6A (●) and the sodium borohydride-reduction product of KC-6A (○).

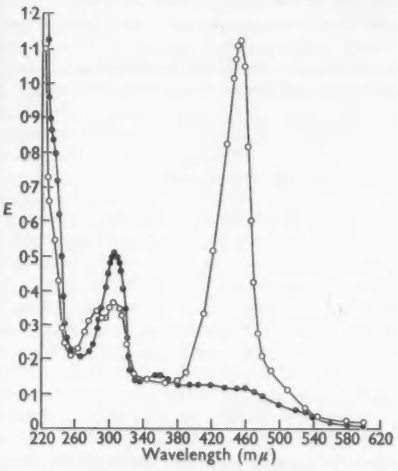

Fig. 2. Absorption spectra, after 2 hr. at 24°, of a solution in sulphuric acid of KC-6A (●) and the sodium borohydride-reduction product of KC-6A (○).

Infrared spectrum. Valuable further preliminary information about the structure of KC-6A was obtained from an infrared spectrum (KCl disk) which was determined by Dr R. K. Callow, National Institute for Medical Research.

In the carbonyl-stretching region there was a prominent band with a somewhat flattened peak at 1725 cm.$^{-1}$ Since Meyer (1955) has reported carbonyl absorption at this frequency with certain 17-oxosteroids in KBr disks it seemed possible that this band in the KC-6A spectrum might be due to a 17-oxo group.

The presence of a band at 1408 cm.$^{-1}$ in the KC-6A spectrum provided support for this view, since Jones & Cole (1952) and Jones, Cole & Nolin (1952) have reported that a band at this frequency in steroids is associated with a 17-oxo group adjacent to an unsubstituted methylene group at C-16.

Jones & Cole (1952), Jones *et al.* (1952) and Kraychy & Gallagher (1957) have ascribed a band at 1375–1377 cm.$^{-1}$ in steroid spectra to the C-13 methyl group. No band at or close to this frequency was seen in the KC-6A spectrum and, accordingly, the possibility that in KC-6A there might be an oxygen substituent at C-18 was further considered.

Action of alkali on KC-6A. At this stage of the investigation the evidence, although far from conclusive, suggested that KC-6A might be 18-hydroxyoestrone.

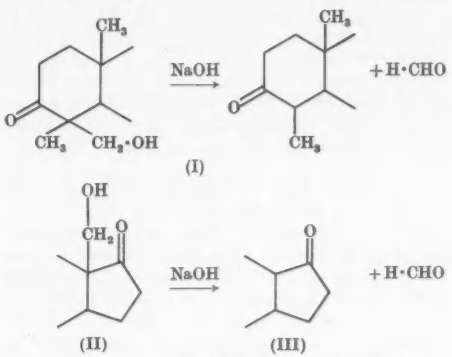

Fig. 3. Action of sodium hydroxide on methyl hederagonate and icterogenin (I, partial formula) (Barton & de Mayo, 1954); hypothetical action of sodium hydroxide on 18-hydroxyoestrone (II, partial formula) with the formation of 18-noroestrone (III, partial formula).

Barton & de Mayo (1954) have shown that the primary β-ketols methyl hederagonate and icterogenin (Fig. 3, I) lose formaldehyde on treatment with alkali at room temperature. It seemed probable therefore that if KC-6A were 18-hydroxyoestrone (Fig. 3, II) it should, by analogy, lose formaldehyde with the formation of 18-noroestrone or its C-13 epimer (Fig. 3, III) on treatment with alkali.

A solution of 100 μg. of KC-6A in 2 ml. of N-NaOH was set aside at room temperature in a stoppered distillation flask. A control flask without KC-6A was also set up. After 4 hr. 3 ml. of 10N-H_2SO_4 and 4 ml. of water were added to each flask and the contents distilled into graduated test tubes each containing 1 ml. of 4% Na_2SO_3 solution. When the volumes of liquid in each tube were 5 ml. the distillations were stopped and the volumes made up to 7 ml. with water. To 2 ml. portions of each distillate was added 3 ml. of 0·2% chromotropic acid in 30N-H_2SO_4 and the mixtures were heated at 100° for 30 min. After cooling the colour developed in the 'experimental' tube was measured at 570 mμ against the 'control', and the amount of formaldehyde corresponding to the colour was read from a previously constructed calibration curve. The yield of the formaldehyde-like substance (9·45 μg.) was 90% of that expected.

The solution remaining in the distillation flask was distributed between water and ether. The ether phase, after washing with 5% $NaHCO_3$ solution and water, was evaporated to dryness. One-fifth of the residue was chromatographed on paper with system 8, when a spot with the same R_F as that of oestrone was detected. A Kober reaction carried out on another portion of the residue was entirely negative.

Since on treatment with alkali KC-6A yielded a volatile substance resembling formaldehyde in nearly the expected amount and a product resembling oestrone in its chromatographic behaviour, KC-6A was provisionally identified as 18-hydroxyoestrone and the oestrone-like product obtained from it as an 18-noroestrone (Loke *et al.* 1957).

Identification of the products formed from KC-6A by the action of alkali

Since the synthesis of 18-hydroxyoestrone did not seem to be immediately practicable it was decided to attempt to obtain proof of the structure of KC-6A by identification of the products formed from it by alkali treatment. At our request, Professor W. S. Johnson of the University of Wisconsin very kindly agreed to attempt the preparation of the two C-13 epimeric 18-noroestrone-3-methyl ethers for comparison with the methyl ether of the oestrone-like compound formed from KC-6A.

From 400 l. of late-pregnancy urine 16 mg. of crude KC-6A was prepared by similar methods to those described above. After three crystallizations from methanol well-formed crystals were obtained which showed a double melting point (about 220° and 248–257°; sealed evacuated capillary).

Material recovered from the mother liquors (11·2 mg.) was dissolved in 200 ml. of N-NaOH and the solution stood at room temperature. After 5 hr. the reaction mixture was acidified with HCl and extracted with chloroform. The extract was washed with 5% Na_2CO_3 solution and water, and dried over anhydrous Na_2SO_4 and evaporated to dryness. The product weighed 10 mg.

Identification of formaldehyde. The acidified aqueous phase from the experiment described above was distilled into 50 ml. of a cold saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl until the total volume was about 150 ml. The mixture was extracted with ether, and the extract washed thoroughly with 10N-H_2SO_4 and water, and dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by filtering in benzene solution through a column of alumina, and then by crystallizing three times from ethanol. The product was obtained in fine yellow needles, m.p. 160–162·5° (hot stage). After admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone (m.p. 162–164°) the m.p. was 161–163°.

Properties and identification of the oestrone-like product

The chloroform-extractable solid obtained from the alkali-treated KC-6A was leached once with 0·2 ml. of cold methanol to remove pigmented contaminants and crystallized from methanol, when 1 mg. of slightly yellow well-formed crystals was obtained, which partially melted with slight decomposition at 263°, resolidified at 265° and finally melted at 266–267° (sealed evacuated capillary). The product gave a negative Kober reaction. The absorption spectrum of a solution in conc. H_2SO_4 after standing for 2 hr. at 24° is shown in Fig. 4. It is noteworthy that this spectrum is virtually superimposable on that given by KC-6A under the same conditions.

The material recovered from the methanol mother liquor (9 mg.) was combined with 2 mg. of a similar product obtained from a previous batch of KC-6A and was methylated with dimethyl sulphate and N-NaOH at 37°. The mixture was extracted with ether and the extract washed with water and evaporated to dryness. The residue failed to give a pure product on crystallization from ethanol. Accordingly it was chromatographed in 33% (v/v) benzene in hexane on 1 g. of alumina. The main fraction from the chromatogram (4·9 mg.) was crystallized twice from methanol, when 1·3 mg.

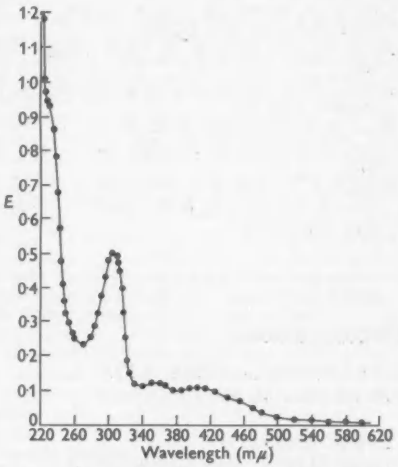

Fig. 4. Absorption spectrum, after 2 hr. at 24°, of a solution in sulphuric acid of the 18-noroestrone obtained from KC-6A by treatment with sodium hydroxide.

of the methyl ether, m.p. 143–147° (hot stage), was obtained.

In the meantime, Professor Johnson and his co-workers had synthesized the two C-13 epimeric DL-18-noroestrone methyl ethers. The mixed m.p. of one of these (m.p. 145–147°, hot stage) with the methyl ether of the product obtained from KC-6A was 142–147°. The infra-red spectra of the two compounds in chloroform solution (kindly determined by Dr W. L. Meyer of the University of Wisconsin) were identical. Accordingly, the oestrone-like compound obtained from KC-6A by the action of alkali is an 18-noroestrone, and it follows that KC-6A was indeed correctly formulated as 18-hydroxyoestrone (Loke *et al.* 1958).

DISCUSSION

Before the present work the only steroids with oxygen substituents at C-18 obtained from animal sources have been aldosterone (11β:21-dihydroxy-3:20-dioxopregn-4-en-18-al) isolated from adrenal glands by Simpson *et al.* (1954), 21-hydroxy-3:20-dioxopregn-4-ene-18-carboxylic acid (as the 18 → 20-hydroxylactone) isolated from adrenal glands by Neher & Wettstein (1956), and 3:20-dioxopregn-4-ene-18:21-diol obtained by Kahnt, Neher & Wettstein (1955) by incubating 11-deoxycorticosterone (3:20-dioxopregn-4-en-21-ol) with adrenal homogenates. Since these compounds are all of adrenal origin it seems reasonable to suppose that 18-hydroxyoestrone may be formed in the adrenal glands by 18-hydroxylation of oestrone.

With this possibility in mind Loke *et al.* (1957) in preliminary experiments incubated oestrone with ox-adrenal homogenates and obtained small yields of a Kober chromogen which resembled 18-hydroxyoestrone in its chromatographic behaviour. On treatment with alkali the product yielded formaldehyde and a substance which resembled oestrone in its chromatographic behaviour. These preliminary findings support the view that 18-hydroxyoestrone may be formed in the adrenal glands.

The fact that sulphuric acid solutions of 18-hydroxyoestrone and of the 18-noroestrone formed from the latter have virtually identical absorption spectra suggests that, under the influence of the strong acid, formaldehyde is eliminated from 18-hydroxyoestrone as it is on alkali treatment. It is reasonable to assume that the same reaction might also occur during the first stage of the Kober reaction, and in this way the fact that 18-hydroxyoestrone is only weakly chromogenic in this reaction could be explained.

SUMMARY

1. A hitherto undescribed ketonic Kober chromogen (KC-6A) has been isolated from the urine of pregnant women in yields of 2–4 mg./100 l.

2. On treatment at room temperature with sodium hydroxide KC-6A yielded formaldehyde and a solid product which was not chromogenic in the Kober reaction but which resembled oestrone in its chromatographic behaviour.

3. This oestrone-like product obtained from KC-6A has been identified (as its methyl ether) as an 18-noroestrone. KC-6A is therefore 18-hydroxyoestrone.

4. 18-Hydroxyoestrone is accompanied in the ketonic–phenolic fractions of pregnancy urine by another Kober chromogen (KC-6B) which has similar chromatographic properties.

The authors gratefully acknowledge the collaboration of Professor W. S. Johnson and his colleagues of the University of Wisconsin which enabled them to bring this work to a successful conclusion. The authors are also indebted to the Medical Research Council and the Melville Trust for Cancer Research for grants from which the expenses of this work were defrayed; to the latter body for a Research Fellowship to K. H. Loke; to Dr R. K. Callow of the National Institute for Medical Research who determined certain infrared spectra; to Dr J. W. Minnis who carried out the microanalyses; and to Mr J. Gordon for his skilled technical assistance.

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Comparative Detoxication

6. THE METABOLISM OF 6-AMINO-4-NITRO-*o*-CRESOL AND 4:6-DINITRO-*o*-CRESOL IN LOCUSTS*

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4:6-Dinitro-*o*-cresol (DNOC) has been used for many years as an insecticide, especially against locust swarms in Africa (Rainey & Sayer, 1953). No chemical data on its metabolism in locusts are available, but it seems possible from toxicological experiments that it is detoxicated in these insects. McCuaig & Sawyer (1957) have shown that repeated sublethal doses of DNOC are not fully cumulative in locusts and point out that this can seriously diminish the effectiveness of field-spraying operations where locusts are sprayed over a period of several days.

Detoxication of DNOC in locusts could be accomplished by a reduction to 6-amino-4-nitro-*o*-cresol, such as is found in rabbits (Smith, Smithies & Williams, 1953), since it is known that locusts can reduce the aromatic nitro group (Friedler & Smith, 1954). We have therefore studied the fate of 6-amino-4-nitro-*o*-cresol in *Locusta migratoria* and *Schistocerca gregaria* as a preliminary to searching for this compound and its metabolites in locusts poisoned with DNOC.

Detoxication of a phenol like DNOC might also occur by conjugation reactions leading to either a β-glucoside or an arylsulphate (cf. Smith 1955*a*, *b*). Locust-crop fluid, however, contains an active β-glucosidase and aryl sulphatase (Robinson, Smith & Williams, 1953), and β-glucosidase has also been found in the intestinal wall of mid- and hind-gut (Robinson, 1956). If these enzymes were to mix with the excretions from the Malpighian tubes, which discharge into the posterior end of the mid-gut of the locust, an initial conjugation might be reversed. We have therefore examined the contents of the different parts of the gut for β-glucosidase and aryl sulphatase in an attempt to assess their possible effect on any *O*-conjugated metabolites before these are eliminated in the excreta.

* Part 5: Smith (1958).

MATERIALS AND METHODS

Reference compounds. DNOC and other phenolic compounds were samples used in earlier work (Smith *et al.* 1953) or prepared by the same methods.

Glucosides of phenols were prepared by the general method of Glazer & Wulwek (1924). Tetra-acetyl-α-glucosyl bromide (34 g.) in 200 ml. of acetone was mixed with 6-acetamido-4-nitro-*o*-cresol (24 g.) in 150 ml. of water containing 6·4 g. of NaOH. After 24 hr. the acetone was removed *in vacuo* at 40–50° and the acetylated glucoside precipitated by addition of water to the residue. The precipitate was crystallized from ethanol to give 5 g. of *6-acetamido-4-nitro-o-cresyl tetra-acetyl-β-D-glucoside* as pale-yellow needles, m.p. 169°; $[\alpha]_D^{25} = -1{\cdot}8°$ (*c*, 1 in $CHCl_3$) (Found C, 51·1; H, 5·0; N, 5·5. $C_{23}H_{28}O_{13}N_2$ requires C, 51·1; H, 5·2; N, 5·2%).

Deacetylation of this compound (5 g.) in 90 ml. of methanol with methanolic 2N-barium methylate (1·5 ml.) at 0° for 18 hr. gave a crystalline precipitate (3·5 g.) which was recrystallized from 90% (v/v) ethanol to give *6-acetamido-4-nitro-o-cresyl β-D-glucoside monohydrate*, m.p. 147–155°; $[\alpha]_D^{25} = -6{\cdot}1°$ (*c*, 0·4 in 50%, v/v ethanol) (Found C, 46·2; H, 6·1; N, 7·7. $C_{15}H_{20}O_9N_2,H_2O$ requires C, 46·2; H, 5·7; N, 7·2%). The water was not lost at 100°. The glucoside had $\lambda_{max.}$ 250, 291; $\epsilon_{max.}$ 11 700, 6900 in 0·1N-NaOH and $\lambda_{max.}$ 240, 289; $\epsilon_{max.}$ 12 500 and 7500 in 0·1N-HCl.

When 6-amino-4-nitro-*o*-cresol was treated as above with acetobromoglucose, two glucosidic derivatives were formed, the major product being an *N*-glycoside. Tetra-acetyl-α-glucosyl bromide (30 g.) in 150 ml. of acetone and 6-amino-4-nitro-*o*-cresol (18 g.) in 50 ml. of water containing 3·2 g. of NaOH were worked up as described above for the acetamido compound and the dark-brown gum was crystallized twice from ethanol to give 5 g. of pale-orange plates: N-(2-*hydroxy*-3-*methyl*-5-*nitrophenyl*) *tetra-acetyl*-β-D-*glucosylamine monohydrate* had m.p. 106°; $[\alpha]_D^{25}$ −69°→ −30° (4 days in $CHCl_3$, *c*, 1) (Found C, 48·5; H, 5·6; N, 5·2. $C_{21}H_{28}O_{12}N_2,H_2O$ requires C, 48·8; H, 5·5; N, 5·4%). This compound could be extracted from solution in $CHCl_3$ into ice-cold 0·1 N-NaOH, as a dark-red solution from which it could be re-extracted with $CHCl_3$ on immediate re-acidification. Treatment with methanolic barium methoxide as described above gave a dark-red amorphous precipitate which was probably the free *N*-glucoside. This was free from barium and very soluble in water but could not be purified without decomposition to the corresponding aminocresol.

The ethanolic mother liquors from the crystallization of the orange-acetylated derivative were diluted with 300 ml. of $CHCl_3$ and washed repeatedly with 0·1 N-NaOH till no further red colour was extracted. The $CHCl_3$ was washed with water, dried with $CaCl_2$ and evaporated *in vacuo* to leave a pale-brown gum which could not be crystallized. This was treated in 50 ml. of methanol with 2 ml. of N-barium methylate for 18 hr. at 0°. On partial evaporation of the methanol, a crop of yellow crystals was obtained which were recrystallized from water to give 300 mg. of 6-*amino*-4-*nitro*-*o*-*cresyl* β-D-*glucoside hemihydrate* as long pale-yellow needles, m.p. 215°; $[\alpha]_D^{25}$ +32·4° (*c*, 0·08 in water); $[\alpha]_D^{25}$ −16·6° (*c*, 0·5 in 0·4 N-HCl) (Found C, 45·5; H, 5·4; N, 8·0; H_2O, 2·8. $C_{12}H_{16}O_8N_2,0{\cdot}5H_2O$ requires C, 46·0; H, 5·6; N, 8·3; H_2O, 2·7%). It had $\lambda_{max.}$ 227, 246, 300, $\epsilon_{max.}$ 11 200, 8700, 5400 in 0·1 N-NaOH and $\lambda_{max.}$ 274, $\epsilon_{max.}$ 7750 in 0·1 N-HCl. Hydrolysis with purified emulsin (L. Light and Co. Ltd.) or boiling N-HCl gave glucose (Myers & Smith, 1954) and 6-amino-4-nitro-*o*-cresol (Table 1) which were identified chromatographically. Acetylation with pyridine and acetic anhydride as described by Kamil, Smith & Williams (1951) gave 6-acetamido-4-nitro-*o*-cresyl tetra-acetyl-β-D-glucoside, m.p. and mixed m.p. 169°.

6-Acetamido-4-nitro-*o*-cresol treated with chlorosulphonic acid and pyridine according to Burkhardt & Lapworth (1926) gave 6-*acetamido*-4-*nitro*-*o*-*cresyl sulphate*, which was isolated as the potassium salt. It formed pale-yellow needles from water (Found C, 33·0; H, 2·7; N, 8·3; S, 10·0; K, 12·1. $C_9H_9O_7N_2SK$ requires C, 32·9; H, 2·8; N, 8·5; S, 9·8; K, 11·9%). It had $\lambda_{max.}$ 240, 287, $\epsilon_{max.}$ 12 400, 7500 in 0·1 N-NaOH and $\lambda_{max.}$ 240 and 285, $\epsilon_{max.}$ 11 100, 7100 in 0·1 N-HCl.

The ethereal sulphate of 6-amino-4-nitro-*o*-cresol was prepared in small yield by the persulphate oxidation of 3-methyl-5-nitroaniline (cf. Boyland, Manson & Sims, 1953). Trial experiments were first carried out with *m*-nitroaniline. *m*-Nitroaniline (10 g.), potassium persulphate (10 g.) and KOH (2 g.) were stirred in a mixture of 100 ml. of acetone and 100 ml. of water for 8 hr. and left overnight. Unchanged *m*-nitroaniline (9·8 g.) was filtered off and the filtrate concentrated to small bulk. The inorganic salts were precipitated by addition of acetone and the filtrate was evaporated *in vacuo* to give a dark-brown residue which was crystallized from ethanol and water. The *potassium* 2-*amino*-4-*nitrophenyl sulphate* (20 mg.) was obtained as dark-yellow needles, very soluble in water, sparingly soluble in ethanol (Found C, 26·9; H, 2·0; K, 14·6. $C_6H_5O_6N_2SK$ requires C, 26·5; H, 1·9; K, 14·4%). It had $\lambda_{max.}$ 270, $\epsilon_{max.}$ 7600 in 0·1 N-HCl, and $\lambda_{max.}$ 225, 245, 288, 355, $\epsilon_{max.}$ 12 200, 10 700, 5300, 2300 in 0·1 N-NaOH.

In a similar experiment 3-methyl-5-nitroaniline (2·5 g.) (Haworth & Lapworth, 1923), potassium persulphate (2·5 g.) and KOH (1 g.) yielded 25 mg. of *potassium* 6-*amino*-4-*nitro*-*o*-*cresyl sulphate* as orange needles, very soluble in water (Found C, 29·6; H, 3·2; K, 14·1. $C_7H_7O_6N_2SK$ requires C, 29·4; H, 2·5; K, 13·7%). In 0·1 N-HCl it had $\lambda_{max.}$ 269, $\epsilon_{max.}$ 6600; in 0·1 N-NaOH it had $\lambda_{max.}$ 225, 293, $\epsilon_{max.}$ 11 700, 4900.

Both ethereal sulphates were rapidly hydrolysed by 0·1 N-HCl at 100° to the corresponding phenols, which were identified by paper chromatography (Table 1).

Locusts. Immature adult *Schistocerca gregaria* or *Locusta migratoria* were obtained from the Anti-Locust Research Centre and kept as previously described (Myers & Smith, 1953). They were fed on grass except while being used for experiment, when they were given water and rice paper.

Compounds were administered to the insects by intrathoracic injection as their sodium salts dissolved in 0·1–0·2 ml. of water. All metabolic experiments described below were carried out on both species and no qualitative difference was observed in the results.

Preparation of extracts. Excreta were roughly ground in a mortar with twice their bulk of aq. 0·1 N-NH_3 soln., then filtered off through cheese cloth and washed with a further equal volume of water. The dark-green filtrate was evaporated to a syrup *in vacuo* at 50° and treated with ethanol until precipitation was complete. The ethanolic filtrate was evaporated to small bulk and a further small amount of insoluble material was thrown down with ethanol. The filtrate was concentrated and used for large-scale chromatography.

Locust tissues were ground with sand in a mortar under ethanol, or, when whole locusts were extracted, macerated in an Ato-Mix Blender with ethanol. An equal volume of water was added and the mixture filtered through cheese cloth. The filtrate was concentrated and the residue precipitated with ethanol as described for excreta extracts, before application to chromatograms.

Paper chromatography. This was carried out as described earlier (Smith *et al.* 1953) and approximate R_F values are quoted in Table 1 to indicate degrees of separation. Identifications were confirmed in all of the solvent systems quoted by running reference compound and unknown on the same paper.

Large-scale chromatography. Crude ethanolic extracts were purified on large-scale paper chromatograms (cf. Smith, 1958), since in most cases the metabolites were associated with large amounts of interfering material and initial chromatograms did not usually give well-defined bands. Zones were located by the yellow or orange colours produced in NH_3 vapour, and cut out and eluted chromatographically with aq. 0·1 N-NH_3 soln. Separation of these initial elutes on further large-scale chromatograms gave a number of well-defined bands, one of which usually predominated. These were eluted for identification.

Paper electrophoresis. This was carried out as described earlier (Smith, 1958), and for identifications the 'unknowns' were run side by side with reference compounds at three or more of the pH values quoted. No significance could be attached to the absolute directions of migration since no allowance was made for electro-osmotic effects. Figures in Table 2 are therefore quoted only to show the degree of separation achieved in a particular electrolyte.

Detection of compounds on papers. All the compounds in Table 1 prevented the fluorescence of paper in ultraviolet light from a filtered low-pressure mercury arc (Hanovia Chromatolite) and showed as dark-purple spots on a lighter background. All nitro compounds except 6-acetamido-4-nitro-*o*-cresyl glucoside gave yellow or orange colours when exposed to NH_3 fumes. Glucosides were detected by hydrolysis to their aglycones on spraying with a 1:5 (v/v) dilution of snail gastric juice, which contains a β-glucosidase, and leaving for 5 min. at room temp. and treating with NH_3 fumes. Ethereal sulphates were detected by hydrolysis to the coloured nitrophenols by spraying with 0·1 N-HCl and heating to 60° for 10 min.

Spectroscopic measurements. These were made in a Unicam SP. 500 spectrophotometer. Eluates of paper chromatograms were measured against controls prepared from a section of paper chromatogram treated as far as possible in the same way as the metabolite spot.

Aryl sulphatase assays. Potassium *p*-nitrophenyl sulphate was used as substrate as described earlier (Robinson, Smith, Spencer & Williams, 1952). A final substrate concentration of 0·033 M in 0·033 M-sodium succinate–succinic acid buffer, pH 5·4, was used for routine assays at 37°.

β-Glucosidase assays. Enzyme solutions (1 ml.) were incubated at 37° with 2 ml. of 0·1 M-phosphate–citrate buffer (prepared from 0·1 M-Na_2HPO_4 and citric acid), pH 7, and 2 ml. of 0·01 M-*p*-nitrophenyl β-glucoside for 0·5 hr.; 5 ml. of 0·2 N-NaOH was then added and the nitrophenol liberated calculated from the absorption at 402 mμ as described for the aryl sulphatase method. Controls were prepared similarly except that the enzyme was added after incubating substrate and buffer, immediately before the addition of NaOH.

RESULTS

Metabolism of 6-amino-4-nitro-o-cresol

Fifty *Schistocerca gregaria*, which were each given 0·4 mg. of 6-amino-4-nitro-*o*-cresol, were decapitated 24 hr. later. The intestinal tract and any adhering organs were removed and an ethanol extract of these was prepared as described above. The final extract was separated on a large-scale chromatogram in solvent *A* (Table 1) in which five yellow or orange zones could be distinguished. The paper was cut into five sections, I–V, corresponding to these zones with approximate R_F values 0–0·15, 0·15–0·3, 0·3–0·5, 0·5–0·7 and 0·7–0·9, and each zone was eluted chromatographically with aq. 0·1 N-NH_3 soln.

Fraction I was chromatographed in solvent *D* and gave a main band with R_F 0·1. This yellow

Table 1. *R_F values of some nitro compounds*

Whatman no. 1 or 4 paper was used and the solvent run until the front had moved 10–12 in. Solvent systems: *A*, Benzene–butanol–aq. NH_3 soln. (sp.gr. 0·88), 2:5:2 (by vol.); *B*, benzene–acetic acid–water, 1:1:2 (by vol.); *C*, butanol–acetic acid–water, 4:1:5 (by vol.); *D*, butanol saturated with water; *E*, ethyl methyl ketone saturated with water; *F*, ethyl methyl ketone–aq. 2 N-NH_3 soln., 1:1 (v/v); *G*, propanol–aq. NH_3 soln. (sp.gr. 0·88), 7:3 (v/v).

	A	*B*	*C*	*D*	*E*	*F*	*G*
6-Amino-4-nitro-*o*-cresol	0·25	0·33	0·91	0·86	0·32	0·47	0·63
6-Amino-4-nitro-*o*-cresyl sulphate	0·49	0·0	0·57	0·51	0·80	—	0·75
6-Amino-4-nitro-*o*-cresyl glucoside	0·70	0·0	0·77	0·60	0·80	—	0·75
6-Acetamido-4-nitro-*o*-cresol	0·53	0·78	0·91	0·82	0·92	0·57	0·75
6-Acetamido-4-nitro-*o*-cresyl sulphate	0·65	0·0	0·56	0·64	0·83	—	0·84
6-Acetamido-4-nitro-*o*-cresyl glucoside	0·70	0·0	0·82	0·69	0·83	—	0·75
3-Amino-5-nitrosalicylic acid	0·06	0·07	0·69	0·26	0·74	0·67	—
3-Acetamido-5-nitrosalicylic acid	0·06	0·01	0·62	0·33	0·56	0·55	—
4:6-Dinitro-*o*-cresol	0·75	0·95	0·95	—	—	0·85	—
2-Amino-4-nitrophenol	0·13	—	—	—	0·95	0·33	—
2-Amino-4-nitrophenyl sulphate	0·25	—	—	—	0·37	0·51	—

Table 2. *Ionophoresis of some nitro compounds*

Apparent migration (in cm.) to anode (+) or cathode (−) on Whatman no. 1 paper in 2 hr. at 12 v/cm.

	0·02 N-HCl	pH 3·9*	pH 6·0*	pH 8·3*	0·02 N-NaOH
6-Amino-4-nitro-*o*-cresol	−3·0	−0·2	+0·1	+0·2	+5·0
6-Amino-4-nitro-*o*-cresyl sulphate	−0·5	+4·3	+4·3	+4·7	+5·2
6-Amino-4-nitro-*o*-cresyl glucoside	−1·5	−0·2	−0·7	−0·1	−1·0
6-Acetamido-4-nitro-*o*-cresol	−0·5	0·0	+0·1	0·0	+4·9
6-Acetamido-4-nitro-*o*-cresyl sulphate	+2·6	+4·6	+4·0	+4·2	+5·0
6-Acetamido-4-nitro-*o*-cresyl glucoside	−0·5	0·0	0·0	0·0	−1·0
4:6-Dinitro-*o*-cresol	−0·5	—	—	—	+5·0

* Britton & Robinson (1931) buffer (British Drug Houses Ltd.).

Table 3. *Absorption spectra in* 0·1 N-*sodium hydroxide of metabolites of* 6-*amino-4-nitro-o-cresol in locusts*

~, Indicates point of inflexion.

	Reference compounds		Metabolites	
	$\lambda_{max.}$	$\epsilon_{max.}$	$\lambda_{max.}$	$E_{1\,cm.}$
6-Acetamido-4-nitro-*o*-cresol	285	6 700	285	0·90
	425	13 900	425	1·52
6-Amino-4-nitro-*o*-cresyl sulphate	225	11 700	225	3·20
	~245	8 500	~245	2·40
	293	4 900	295	1·28
6-Amino-4-nitro-*o*-cresyl glucoside	227	11 200	230	3·52
	247	8 700	~245	3·05
	295	5 400	295	1·68

material, which was also present in extracts of untreated locusts, was not identified. No 3-amino-5-nitrosalicylic acid or 3-acetamido-4-nitrosalicylic acid, which have R_F values near 0·1 in solvent *A*, was detected in this extract. Small amounts of material corresponding to the main bands of fractions II–V were also present.

Fraction II was chromatographed in solvent *D* and gave two main zones. A pink band of R_F 0·4, which on elution had light-absorption maxima at 460 and 270 mμ in 0·1 N-NaOH and at 320 and 270 mμ in 0·1 N-HCl, was not identified. A second major, orange band in solvent *D*, with R_F 0·8, was identified as 6-amino-4-nitro-*o*-cresol by paper chromatography, absorption spectrum in 0·1 N-NaOH and electrophoresis. From the extinctions in 0·1 N-NaOH it was calculated that 5% of the dose was present in this form and in other experiments corresponding values ranged from 5 to 8%.

Fraction III was chromatographed in solvent *D* and gave a pale-yellow main band of R_F 0·5, which was eluted with aq. 0·1 N-NH_3 soln. and identified as the ethereal sulphate of 6-amino-4-nitro-*o*-cresol by paper chromatography, ionophoresis and comparison of absorption spectra (Table 3). It was rapidly hydrolysed by boiling 0·1 N-HCl to 6-amino-4-nitro-*o*-cresol. Calculation from the absorption spectrum in 0·1 N-NaOH showed that 0·5% of the dose was present as this ethereal sulphate.

Fraction IV was chromatographed in solvent *D* and gave a major yellow band which after elution was identified as 6-acetamido-4-nitro-*o*-cresol by chromatography, ionophoresis and measurement of absorption spectrum (Table 3). From the absorption spectrum in 0·1 N-NaOH it was calculated that 0·2% of the dose was present in this form and in other experiments the corresponding values ranged from 0·2 to 5·0% of the dose. A small amount of material of R_F 0·6 was pooled with fraction V.

Fraction V was chromatographed in solvent *D* and gave a main, pale-yellow band of R_F 0·6, which was eluted and identified as 6-amino-4-nitro-*o*-cresyl glucoside by paper chromatography, ionophoresis and measurement of absorption spectrum in 0·1 N-NaOH. From this spectrum it was calculated that 0·7% of the dose was present as the glucoside.

Extracts of *Locusta migratoria* dosed with 6-amino-4-nitro-*o*-cresol were fractionated as described above and 6-acetamido-4-nitro-*o*-cresol, 6-amino-4-nitro-*o*-cresol, its glucoside and its ethereal sulphate were identified as with *Schistocerca*.

These four compounds were also separated and identified in extracts of *Schistocerca* and *Locusta* made 4 hr. after dosing, and at this time the hind-gut of some locusts was full of a red liquid in which these compounds could be directly demonstrated by paper chromatography.

In some experiments excreta were collected for 48 hr. after dosing and extracts made as described above. The same metabolites were present in these extracts but the amounts of *O*-conjugates, especially the glucoside, were much smaller. This is probably attributable to the variable but usually large amounts of β-glucosidase in the hind-gut and excreta (see below).

Metabolism of 6-*acetamido-4-nitro-o-cresol*

Extracts were made as described above after dosing 100 locusts with 0·25 mg. each of 6-acetamido-4-nitro-*o*-cresol. After fractionation of visceral extracts as described above, 2% of unchanged material was recovered from the chromatograms. An additional 12% of the dose was recovered by similarly extracting the eviscerated bodies, but no *O*-conjugates could be detected in this or a number of other experiments with *Locusta* and *Schistocerca*.

Metabolism of 4:6-*dinitro-o-cresol*

Two hundred *Locusta migratoria* which had received 4 μg. each of DNOC were decapitated 24 hr. later and an ethanol extract was prepared from the combined viscera and excreta. The final extract was separated on a large-scale chromatogram in solvent *A*. A feeble-yellow band at R_F 0·7 was eluted and rechromatographed in solvent *F* to

free it from a large amount of fast-running brown waxy material. The yellow band with R_F 0·2 was eluted with aq. 0·1 N-NH_3 soln. and identified as 6-acetamido-4-nitro-*o*-cresol by chromatography, ionophoresis in 0·02 N-NaOH and comparison of absorption spectra (Fig. 1). The extinction in 0·1 N-NaOH corresponded to a recovery of 1% of the dose. When boiled with N-HCl for 1 hr. the metabolite was converted into 6-amino-4-nitro-*o*-cresol, which was extracted with ether and identified chromatographically. 6-Acetamido-4-nitro-*o*-cresol was similarly identified in parallel experiments with *Schistocerca gregaria*.

In another experiment 22 μg. of DNOC was given to each of 24 *Schistocerca* in two divided doses with a 3 hr. interval, and an extract of viscera made 3 hr. later. At this time a trace of DNOC could still be detected in the haemolymph by paper chromatography.

Separation of the visceral extracts on an initial chromatogram in solvent *A* gave, as before, a yellow band at R_F 0·7. On rechromatographing this in solvent *F* it separated into a minor band identified, as above, as 6-acetamido-4-nitro-*o*-cresol (<1% of dose) and a more definite band which was identified by paper chromatography and measurement of absorption spectra as DNOC (4% of dose).

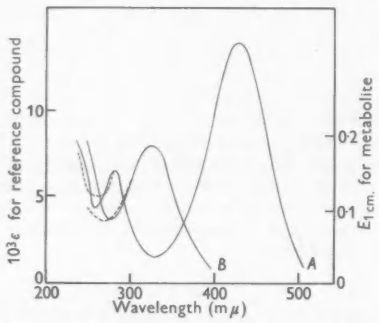


Fig. 1. Absorption spectra of 6-acetamido-4-nitro-*o*-cresol (continuous lines) and of metabolite from locusts poisoned with DNOC (broken lines). For wavelengths between about 300 and 500 mμ the curves for reference compound and for metabolite coincided. Curves *A*, in 0·1 N-NaOH; curves *B*, in 0·1 N-HCl.

Enzyme experiments

Distribution of glucosidase and sulphatase in gut. The heads and terminal abdominal segments of locusts were cut off and the intestinal tracts removed. The gut was divided immediately anterior to the attachment of the gastric caecae and at the point of attachment of the Malpighian tubes. The three sections, designated fore-gut, mid-gut and hind-gut, were opened, and the dark-coloured contents weighed and diluted to measured volume with water. The mid-gut solution included the contents of the gastric caecae. Freshly voided, moist excreta were also weighed, and suspended in a measured volume of water; all solutions were diluted to a volume containing enough activity to give spectrophotometer readings of 0·2–1·0 and were filtered through cotton wool before use.

(*a*) Arylsulphatase activity in each section of gut showed optimum activity in succinate buffer at pH 5·4. Optimum substrate concentration was not reached with saturated solutions of potassium *p*-nitrophenylsulphate. Plots of $1/V$ against $1/S$ (cf. Alberty, 1956) for the range 0·5–5·0 mM were linear and crossed the $1/S$ axis close to the origin at values corresponding to K_s values of not less than 0·05 M.

(*b*) β-Glucosidase activity in each section of the gut showed a broad maximum between pH 5 and 6 and there was little loss of activity at pH 7 in phosphate–citrate buffer. Measurements were carried out at pH 7 since this was the pH of moist excreta, and the optimum substrate concentration determined empirically at this pH was 4 mM-*p*-nitrophenyl glucoside. Plots of $1/V$ against $1/S$ for the range 0·4–4·0 mM were linear and the intercept on the $1/S$ axis corresponded to values of K_s of 0·9 mM for each section of the gut.

Small groups of *Schistocerca* were obtained from the Anti-Locust Research Centre at different times and the glucosidase and sulphatase activity of the sections of the gut measured. The results are summarized in Table 4. Locusts in these experi-

Table 4. *Enzymic liberation of* p-*nitrophenol from conjugates by gut contents and excreta of* Schistocerca gregaria

For conditions see Methods. Ranges of values are given in parentheses.

	Fore-gut	Mid-gut	Hind-gut	*Excreta
From *p*-nitrophenyl sulphate				
Total μg./hr.	590 (70–2700)	210 (16–860)	11 (0–40)	3 (0–10)
μg./mg. of gut contents/hr.	20 (2–40)	7 (1–17)	0·5 (0·1–1)	0·2 (0–0·9)
From *p*-nitrophenyl glucoside				
Total μg./hr.	510 (220–710)	390 (80–980)	330 (60–650)	767 (114–1600)
μg./mg. of gut contents/hr.	30 (8–45)	32 (2–180)	24 (14–130)	120 (12–220)

* Freshly voided faecal pellets, total wt. 2–10 mg. were used.

ments were fed, as normally, on grass but no significant diminution of activity occurred in one experiment where a group of locusts was starved for a week before assay. Similar high β-glucosidase activity was found in *Locusta* gut contents.

Hydrolysis rates of glucosides

Solutions of 6-amino-4-nitro-*o*-cresyl glucoside and 6-acetamido-4-nitro-*o*-cresyl glucoside were rapidly hydrolysed to the corresponding phenols when ground at room temperature with dry excreta from the stock cage. On the other hand, *m*-aminophenyl glucoside has been separated fairly readily from excreta (Myers & Smith, 1954) and was therefore presumably fairly stable. We have therefore measured the hydrolysis rates of some of the glucosides with enzyme from excreta.

Solutions of glucosides, 3 ml. of 0·01 M in 0·02 M-phosphate buffer (prepared from 0·02 M-Na_2HPO_4 and 0·02 M-KH_2PO_4), pH 7, and enzyme (1 ml.), prepared by grinding 5 g. of dry excreta with 25 ml. of water and centrifuging the debris, were incubated at 37°. Samples of this solution and similar controls prepared with boiled enzyme were withdrawn at intervals and analysed for the aglycones. Control samples were used as instrument blanks in the spectrophotometer. Nitrophenols were estimated by their characteristic absorption in 0·1 N-NaOH (see above). *m*-Aminophenol was estimated, after ether extraction, by the dimethylaminobenzaldehyde method used earlier for *m*-aminophenyl sulphate (Smith, 1955*b*).

With one sample of enzyme the values of $t_{\frac{1}{2}}$ calculated from the unimolecular velocity constant were 31 min. for 6-amino-4-nitro-*o*-cresyl glucoside, 33 min. for *p*-nitrophenyl glucoside, 85 min. for 6-acetamido-4-nitro-*o*-cresyl glucoside and 700 min. for *m*-aminophenyl glucoside.

DISCUSSION

The results quoted in Table 4 show that the β-glucosidase activity which occurs in locust-crop fluid is found in all the other parts of the intestine (cf. Robinson, 1956) and is also present in substantial amounts in the excreta. It is possible that the activity is due to the crop enzyme being carried along with the food. The hind-gut in a number of locust species has a pH of 7–8 (Uvarov, 1948), and at this value the β-glucosidase is still active. It is probable that a considerable part of any β-glucoside excreted from the Malpighian tubes is hydrolysed before elimination in the excreta, but the extent of this hydrolysis will depend on the nature of the glucoside as well as on the amount of activity present in the gut. 6-Amino-4-nitro-*o*-cresyl glucoside is hydrolysed about 20 times as rapidly as *m*-aminophenyl glucoside. It is likely, however, that sufficient glucosidase is always present in hind-gut to hydrolyse completely any glucoside produced from the very small doses of DNOC used, even if all the insecticide had been converted into its β-glucoside.

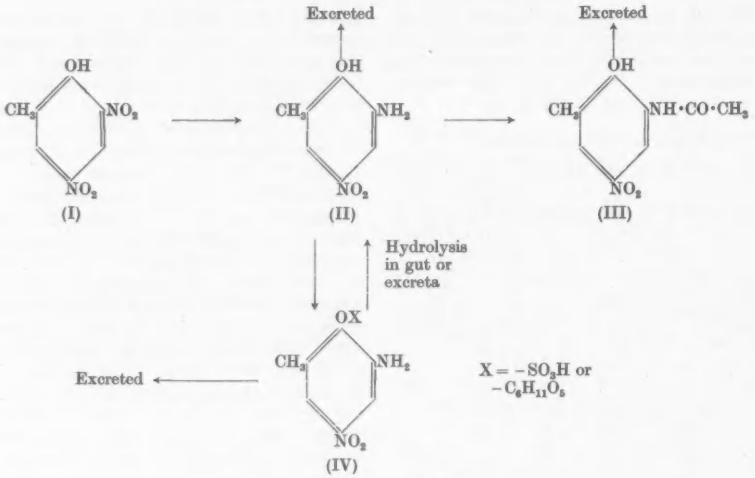
It has already been pointed out that a glucuronic acid conjugation would be of small value to a locust because of the presence of β-glucuronidase (Smith, 1955*b*), and the same observation must now be applied to the β-glucoside conjugation. The importance to the locust of this hydrolysis of conjugates in the gut must largely depend on how well the liberated phenols can be reabsorbed from the small intestine; it is possible that locusts recycle excreted phenols after gut hydrolysis of the initial conjugate. A similar situation occurs in mammals, where some compounds, e.g. chloromycetin (Glazko, Dill & Wolf, 1952), are excreted as conjugates in the bile, and hydrolysed in the intestine and reabsorbed into the system. This situation is more serious for the locust, however, as no alternative (urinary) route of excretion is available. If this recycling of phenols does occur in locusts the toxicity of phenols might be expected to be greater in non-feeding insects since the gut contents would not then be so rapidly expelled (cf. Uvarov, 1948).

The association of β-glucoside conjugation with active β-glucosidase activity may be fairly general. The glucoside conjugation appears fairly general in insects (Smith, 1955*a*) and β-glucosidase activity has been demonstrated in a variety of insects (Robinson, 1956, 1957), and in a number of species this is localized in the intestine (Newcomer, 1954; Evans, 1956; Koike, 1954).

Distribution of arylsulphatase activity in the locust intestine differs from that of β-glucosidase in that much less is found in hind-gut than in crop and mid-gut. Moreover, *p*-nitrophenyl sulphate requires a high substrate concentration for optimum activity. It may be for this reason that we were able to detect 6-amino-4-nitro-*o*-cresyl suphate in extracts of locust excreta after dosing with the cresol, and in many experiments the corresponding glucoside was not detectable.

The presence of these two enzymes in the gut makes it unlikely that any effective detoxication of DNOC occurs by *O*-conjugations and no such metabolites could be detected in the poisoned locusts. Reduction of a nitro group appears to be the effective detoxication in locusts as in rabbits. The metabolism of DNOC (I) and 6-amino-4-nitro-*o*-cresol (II) is shown in scheme 1.

When 6-amino-4-nitro-*o*-cresol was injected, the acetyl derivative (III) and the two *O*-conjugates (IV) were identified, but when DNOC was injected, necessarily at a lower dose level, only 6-acetamido-4-nitro-*o*-cresol (III) was found as a metabolite.



Scheme 1

SUMMARY

1. 6-Amino-4-nitro-*o*-cresol is conjugated in the locusts *Schistocerca gregaria* and *Locusta migratoria* to 6-amino-4-nitro-*o*-cresyl glucoside and 6-amino-4-nitro-*o*-cresyl hydrogen sulphate, which have been identified in the gut and excreta by chromatographic, ionophoretic and spectroscopic methods.

2. Small amounts of 6-acetamido-4-nitro-*o*-cresol were similarly identified in extracts of locusts poisoned with 4:6-dinitro-*o*-cresol.

3. β-Glucosidase activity is present in the contents of locust fore-, mid- and hind-gut, and in locust excreta.

4. Aryl sulphatase activity is present in locust fore-gut and mid-gut contents, and to a much smaller extent in hind-gut and excreta.

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Formation of Collagen Hydroxyproline *in vitro*

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Stetten & Schoenheimer (1943) fed [^{15}N]proline to young rats and showed it to be a good source of the hydroxyproline of the carcass protein. Later, Stetten (1949) found that the efficiency of dietary [^{15}N]hydroxyproline was less than one-twentieth of that of proline as a precursor of collagen hydroxyproline. These findings are of considerable interest since they are contrary to the general observation that proteins are built up from their constituent free amino acids (Loftfield, 1957). The only close parallel, discovered subsequently, is the formation of collagen hydroxylysine from lysine (Sinex & Van Slyke, 1955; Piez & Likins, 1957), but not from free hydroxylysine (Sinex & Van Slyke, 1957). Stetten (1949) suggested that free hydroxyproline was not an intermediate in collagen biosynthesis and that hydroxylation of the proline residues occurred at the protein or peptide stage. The hydroxylation of an unreactive aliphatic carbon atom is a reaction which would be expected to require some form of preliminary activation of the substrate and although it is not impossible that this might take place in a protein it appears much more likely that a low-molecular-weight substrate would be involved. However, a number of workers (Robertson & Schwartz, 1953; Gould & Woessner, 1957) have endeavoured to obtain evidence for a collagen-like protein intermediate lacking the hydroxyl groups of hydroxyproline in the tissues of scorbutic guinea pigs. They assumed that the impaired collagen synthesis in these animals resulted from inability to hydroxylate such an intermediate protein. It was shown that administration of vitamin C to scorbutic animals led to increases in hydroxyproline and parallel decreases in proline in collagen-forming tissues, but since no isolation of the hypothetical precursor was attempted the results are not conclusive.

This work was undertaken with the object both of studying the hydroxylation of proline in relation to collagen formation and of analysing this process in terms of current theories of protein synthesis. In order to obtain information about intermediates it was decided to use the granuloma induced in guinea pigs by subcutaneous injections of carrageenin (Robertson & Schwartz, 1953), since this tissue synthesizes collagen rapidly *in vivo* and appeared to be a promising tissue for experiments *in vitro*. Moreover, Jackson (1957) had already studied the incorporation *in vivo* of labelled glycine into the neutral-salt-soluble, citrate-soluble and insoluble collagen fractions of this tissue and had confirmed the results of Harkness, Marko, Muir & Neuberger (1954) for the corresponding fractions from rabbit skin. We have based our approach on the work of these authors, using labelled proline and hydroxyproline in place of glycine and using tissue slices instead of whole animals.

MATERIAL AND METHODS

Carrageenin. This was a commercial dried extract of Irish Moss obtained from the Seaplant Chemical Corporation, New Bedford, Mass., U.S.A. and was used without further purification.

Amino acids. Commercial L-proline was found to contain 3–4% of hydroxyproline, which could not readily be removed by recrystallization. A pure sample was prepared from the mixed reineckates of proline and hydroxyproline, which had been obtained from a gelatin hydrolysate (Neuberger, 1945). The reineckates were dissolved in 30% (v/v) methanol and passed through a column of Zeo-Karb 225 resin in the hydrogen form. The reinecke acid emerged in the effluent and after washing the column the proline and hydroxyproline were eluted with aq. N-NH_3 soln.

After removal of the NH_3 by evaporation the two imino acids were separated from each other and from small amounts of other amino acids by displacement chromatography on Zeo-Karb 225 resin (200-mesh) with aq. 0·1N-NH_3 soln. as the displacing agent (Partridge & Westall, 1949). The pooled proline fractions contained less than 0·5% of hydroxyproline.

Unlabelled L-hydroxyproline was obtained from British Drug Houses Ltd. It contained less than 0·2% of proline and was chromatographically pure.

Uniformly labelled L-[^{14}C]proline was obtained from The Radiochemical Centre, Amersham, Bucks. Inactive L-proline was added to give a specific activity of 73 000 counts/min./μmole (0·47 μc/μmole). Chromatography in butan-1-ol–acetic acid–water (63:10:27) gave a single spot on developing with ninhydrin, and radioautography of the chromatogram showed that apart from the proline spot there was a faint spot in the valine position and a very faint streak extending from the proline spot towards the origin. This contamination was regarded as negligible.

Uniformly labelled L-[^{14}C]hydroxyproline was prepared from combined neutral-salt-soluble collagen and acid-soluble collagen extracted from granuloma slices incubated with labelled proline and isolated as described below. Tissue slices (20 g.) were incubated with 40 μc of uniformly

labelled proline ($5{\cdot}4 \times 10^6$ counts/min.) and from the hydrolysed soluble collagens 1·4 mg. of hydroxyproline was isolated, having a specific activity of 5600 counts/min./μmole.

Chemical estimations. Hydroxyproline was determined by the method of Neuman & Logan (1950) and proline by the ninhydrin method of Troll & Lindsley (1955). Raffinose was estimated by the orcinol method of Roe, Epstein & Goldstein (1949) and urea by the determination of NH_3 released during incubation with urease as described by Conway (1950). Total N was estimated by the Kjeldahl method. Approximate estimations of amino N were made by the colorimetric ninhydrin method of Meyer (1957).

Granuloma production. Male albino guinea pigs weighing 400–500 g. were shaved on the abdomen and injected subcutaneously with 5 ml. of a 1% solution of carrageenin in 0·9% NaCl soln. during ether anaesthesia. At the required interval after the injection the animals were killed by a blow on the neck and the granulomata were peeled away from the abdominal muscle and dermis and chilled in cold saline. The wet weight varied between 10 and 20 g.

The 7-day-old granuloma has been used for most of the metabolic experiments, since Jackson (1957) showed that the rate of collagen synthesis reached its maximum between the sixth and seventh day. At this stage the tumour weighed from 10 to 25 g. and consisted of a central gelatinous region containing little collagen, but large numbers of blood cells suspended in fluid dispersed throughout the gel. The paper-electrophoretic pattern of the fluid expressed from the gel was similar to that given by guinea-pig serum. This central region, occupying perhaps half of the bulk of the granuloma, was surrounded and penetrated by bands of highly cellular fibrous tissue which on the dorsal surface contained variable amounts of muscle derived from the panniculus carnosus.

Incubation of granuloma slices. Blocks of tissue were cut from the granuloma with scissors and 0·5 mm. slices were cut on a Stadie–Riggs microtome (Stadie & Riggs, 1944). Slices cut from the dorsal surface were often contaminated by muscle fibre and were rejected; the rest were kept at 0° in a beaker packed in ice chips.

For isotope experiments 10 g. wet wt. of slices was transferred into 50 ml. of ice-cold phosphate saline medium 4A of Krebs (1950) in a 250 ml. conical flask. The organic constituents were replaced by 22·4 mM-glucose and the following concentrations of amino acids: L-leucine (0·76 mM), L-isoleucine (0·38 mM), L-valine (0·85 mM), L-threonine (0·84 mM), L-methionine (0·13 mM), L-arginine (0·95 mM), L-lysine (1·1 mM), L-tyrosine (0·22 mM). After the addition of labelled proline the flasks were gassed with O_2 and stoppered and incubated at 37°.

In experiments designed to estimate the distribution of proline or hydroxyproline between the slices and the medium the amino acid supplements were omitted and the glucose concentration was reduced to 11·2 mM in order to reduce interference by glucose in the subsequent estimation of raffinose.

Intracellular and extracellular distribution of proline and hydroxyproline when incubated with slices of granuloma. Tissue slices were incubated in the presence of urea, raffinose, L-proline and L-hydroxyproline and after time for equilibration they were separated from the medium. The concentration (c) of each substance in the medium and the total amount (m) in the slices were then determined. The quantity m/c then gives the fluid space which the substance would occupy if it were present in the slices at its extracellular concentration. Since urea distributes itself uniformly between extracellular and intracellular water, the urea space is equal to the total slice water. Raffinose on the contrary does not enter the cells (Helmreich & Cori, 1957) and the raffinose space is therefore equal to the extracellular water. The intracellular water is thus obtainable by difference. The extracellular imino acid in the slice is given by the product of its concentration in the medium and the raffinose space. The amount of intracellular imino acid is then obtained by subtracting this from the total slice imino acid. The intracellular concentration can then be calculated since the amount of intracellular water is known.

About 1 g. wet wt. of granuloma slices was added to 5 ml. of ice-cold medium supplemented with L-proline (3·47 mM) or L-hydroxyproline (3·05 mM), glucose (11·2 mM), raffinose (6·7 mM) and urea (10 mM). The flasks, set up in triplicate or quadruplicate, were stoppered and incubated in air at 37° for 35 min. with shaking and cooled in ice-water for a few minutes. The slices were removed from the supernatant with a glass hook and allowed to drain on the sides of the flask.

The drained slices were weighed in tared specimen tubes, transferred to a small mortar and ground with 1 g. of sand and 2 ml. of saturated picric acid solution. After adding a further 8 ml. of saturated picric acid solution the protein precipitate was centrifuged and a sample (8 ml.) of the supernatant passed down a 1·6 cm. × 1·0 cm. column of Dowex-2 (200-mesh) in the chloride form to remove picric acid. The effluent and washings were made up to 15 ml.

The supernatant from the incubation flask was centrifuged for 5 min. to remove debris and a 5 ml. sample deproteinized with 5 ml. of saturated picric acid solution. A sample (8 ml.) of the supernatant was passed through a similar column of Dowex-2 and the effluent and washings were made up to 15 ml. The total volume of the supernatant was obtained from the following expression: vol. of medium added plus loss in weight of slices during incubation minus dry weight of protein in the supernatant. Proline, hydroxyproline, urea and raffinose were estimated in suitable fractions of the 15 ml. effluent. In each experiment tissue blanks were obtained by incubating slices in suspending medium without supplements. Recoveries were measured by the addition of known amounts of L-proline, L-hydroxyproline urea and ruffinose to the separated slices and supernatant immediately before deproteinization. With this technique, quantitative recoveries (96–99%) were obtained. When trichloroacetic acid was used to precipitate protein the imino acid recoveries were low.

Determination of free proline and hydroxyproline in granuloma slices. The method was essentially the same as that described above for the tissue blanks except that (i) double quantities of tissue and medium were used; (ii) the slices were separated and washed by centrifuging as in the L-[^{14}C]proline-incorporation experiments described in the next section; (iii) the picric acid was removed by taking up the amino acids on Zeo-Karb 225 (200-mesh), and washing away the picric acid and eluting with aq. N-NH_3 soln. This last procedure had the effect of concentrating the solution, since the volume of NH_3 soln. eluate was quite small.

Proline and hydroxyproline were determined after evaporating the NH_3 and taking up in a small volume of

water. On account of the small amounts of hydroxyproline to be determined the Neuman & Logan method was scaled down by a factor of two.

Estimation and purification of collagen from granuloma slices. After incubation the flasks were chilled in ice–water and the contents centrifuged at 0°. The supernatant was removed and the packed slices were rapidly washed by suspending in cold medium and again centrifuging. The packed slices and the combined supernatant and washings were stored at −20°.

To obtain a finely divided tissue suitable for extraction of soluble collagen the frozen packed slices were crudely sliced and dropped into acetone–solid CO_2. The brittle slices were blotted free from acetone and powdered in a mortar previously cooled with the acetone–solid CO_2 mixture. The powder was transferred to a chilled 50 ml. centrifuge tube and allowed to stand at 0° for a few minutes to enable any CO_2 to evaporate before extracting soluble collagen. The extraction procedure was based on that used by Jackson (1957) and is shown in Fig. 1. All operations were carried out at 4°.

The powder was washed twice with 15 ml. of water to extract free tissue amino acids. Trichloroacetic acid was added to the extract to a concentration of approx. 20% and proline and hydroxyproline were isolated as described below. Since this water extract might also have contained a metabolically active water-soluble collagen, the trichloroacetic acid precipitate was heated for 30 min. with 5 ml. of hot 5% (approx.) trichloroacetic acid to extract gelatin (Fitch, Harkness & Harkness, 1955). The trichloroacetic acid was extracted with ether and the solution concentrated to give water-soluble fraction *A*.

The washed powder was extracted overnight with four successive portions of 0·2 M-NaCl soln. buffered with 7 mM-ethylenediaminetetra-acetic acid (EDTA), pH 7·4 (15 ml./10 g. wet wt. of tissue). The combined extracts contained the neutral-salt-soluble collagen. This was precipitated by adding solid NaCl to a final concentration of 16% and the precipitate centrifuged at 26 000 *g* in the Spinco Model L Ultracentrifuge for 30 min. This precipitate was redissolved in 0·2 M-NaCl soln., pH 7·4, to which a little inactive proline had been added, and dialysed overnight against two changes of 2 l. of 0·2 M-NaCl soln. buffered with 0·01 M-phosphate, pH 7·4. The collagen was again precipitated by adding an equal volume of cold 30% (w/v) NaCl soln. and the precipitate centrifuged at 26 000 *g*. This precipitate was gelatinized by heating to 80° in a small volume of water.

The supernatant from the first precipitation of neutral-salt-soluble collagen contained the water-soluble collagen fraction described by Jackson (1957). The NaCl was removed by dialysis against large volumes of water in the cold, and the precipitated protein was spun down. The supernatant was concentrated and the gelatin extracted by heating to 90° with 5% trichloroacetic acid. The trichloroacetic acid was removed by ether extraction and the solution concentrated to give water-soluble fraction *B*.

The residue after extraction of neutral-salt-soluble collagen was then extracted overnight with three successive 10 ml. portions of sodium citrate–HCl buffer (0·5 M; pH 3·8; Britton, 1955), and finally with 10 ml. of sodium acetate buffer (0·2 M; pH 3·8; Britton, 1955). Citrate-soluble collagen was precipitated from the combined extracts by adding solid NaCl to a final concentration of 5%. The precipitate was centrifuged in the Spinco Ultracentrifuge as before and the supernatant was rejected. The acid collagen was then redissolved in 3–4 ml. of 0·01 N-acetic acid containing about 10 mg. of proline and dialysed for 24 hr. against 2 × 2 l. of 0·01 M-sodium and potassium phosphate buffer, pH 7·4. After dialysis the sac content was spun as before and the precipitate was gelatinized in water at 80°.

Insoluble collagen was isolated from the alkali-washed residues as described by Jackson (1957). All the collagen fractions were hydrolysed at 110° with 6 N-HCl for 18 hr. in sealed tubes.

Extraction of free proline and hydroxyproline. The free hydroxyproline and proline present in the supernatant from the incubation and in the water washings of the powdered frozen slices were isolated after deproteinization with cold 20% trichloroacetic acid. The trichloroacetic acid supernatants were extracted three times with an equal volume of ether to remove trichloroacetic acid and then the amino acids were desalted and chromatographed as described below.

Desalting and chromatography. All amino acid solutions were desalted before chromatography and before counting on short columns of Zeo-Karb 225 (200 mesh) in the hydrogen form. The amino acid solution was run on to a column containing about 1 g. of resin (or more if a large amount of salt was present) and the column was washed with three or four volumes of water. Amino acids were eluted with aq. N-NH_3 soln. and the eluates taken to dryness *in vacuo*.

One-dimensional chromatograms were run in the following solvent systems: (1) water-saturated phenol in an atmosphere of ammonia; (2) butan-1-ol–acetic acid–water (63:27:10); (3) pyridine–amyl alcohol–water (35:35:30). Two-dimensional chromatograms were run in the first two of these in succession.

Isolation of proline and hydroxyproline. The hydrolysates were dried *in vacuo*, dissolved in water and when necessary the solutions were desalted. The dried eluates were redissolved in 0·2 ml. of water and applied as a streak to sheets of Whatman no. 3 MM paper. Marker spots (containing 10 μg. of proline, hydroxyproline, glycine and leucine) were applied 3 cm. from either end of the streak and a descending chromatogram was developed for 27 hr. in phenol. The chromatogram was then dried at 60–70° in a current of air and the positions of the proline and hydroxyproline were detected in strips cut in the direction of the run, so as to include the marked spots and approx. 1 cm. of the edges of the streak. These strips were dipped in a 0·1% solution of ninhydrin in acetone and air-dried, and the proline and hydroxyproline revealed as pink and red spots respectively by steaming over a boiling-water bath (Clarkson, 1955). Other amino acids were detected by subsequent heating in the oven. Strips corresponding to the proline and hydroxyproline were then cut from the rest of the chromatogram and the amino acids eluted with water as in a descending chromatogram. The concentrated eluates were rechromatographed with the same technique in butanol–acetic acid–water for 36 hr., and the proline and hydroxyproline were detected and eluated as before. Imino acids isolated from collagen hydrolysates were usually found to be free from other ninhydrin-reacting material at this stage. However, chromatographed extracts of tissue amino acids or supernatant amino acids often showed contamination with small amounts of other ninhydrin-reacting material and therefore a further chromatogram

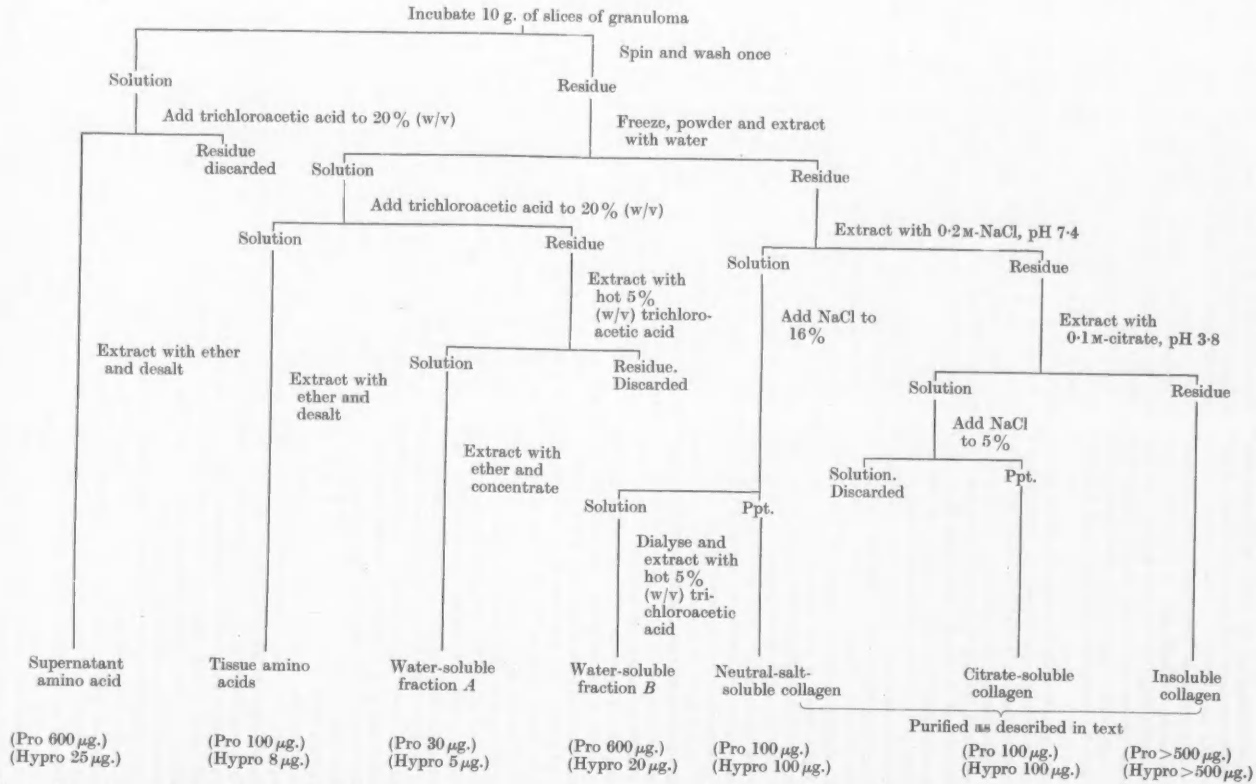

Fig. 1. Extraction and purification of amino acids and collagen fractions from slices of granuloma incubated with labelled amino acids. The quantities in parentheses refer to the approximate amounts (Pro, proline; Hypro, hydroxyproline) isolated from each fraction for counting.

was run in the pyridine–amyl alcohol–water system, or the nitrous acid procedure described below was used.

In later experiments the phenol chromatogram was omitted and the hydrolysate was first treated with nitrous acid which deaminated all the α-amino acids to the corresponding α-hydroxy acids. After this a single run in the butanol–acetic acid–water usually sufficed to separate proline and hydroxyproline in a chromatographically pure state.

Isolation of glutamic acid. In one experiment glutamic acid was isolated from the phenol chromatogram of the free amino acid fractions, and desalted and counted as described below.

Nitrous acid treatment of the mixed amino acids. The method adopted was based on that of Schweet (1954). Ice-cold sodium nitrite solution (3 ml. of 40%, w/v) was pipetted slowly beneath the surface of 10 ml. of ice-cold conc. HCl and the precipitated NaCl allowed to settle. Samples of the brown supernatant were transferred by Pasteur pipette to the amino acid solution in a test tube, in the ratio of 1·3 ml. to approx. 20 mg. of amino acid mixture, and the reaction mixture was immediately shaken in an oil bath at 120° for 2·5 min. The solution was cooled and evaporated to dryness in a desiccator. Proline and hydroxyproline were desalted and chromatographed in butanol–acetic acid–water as previously described. This procedure gave clean bands of proline and hydroxyproline both from collagen hydrolysates and from tissue and supernatant amino acid fractions. However, a second butanol–acetic acid–water run was necessary for the hydroxyproline from the amino acid fractions since very slight trailing of the strongly radioactive proline band gave rise to large errors in the specific activity of the hydroxyproline, which contained only about 0·1% of the total proline counts.

This method gave 80–90% recovery of proline and hydroxyproline from collagen hydrolysates. The recovery of small amounts of hydroxyproline from large amounts of other amino acids was much lower (about 50%).

Counting of isolated proline and hydroxyproline. All amino acid samples after elution from the chromatogram were desalted before plating for counting. This was necessary since between 2 and 4 mg. dry wt. of material could be extracted from strips of paper of the size normally used and this was sufficient to introduce considerable self-absorption errors when counting at infinite thinness. The use of paper washed with acetic acid or water for chromatography was not satisfactory since the washing greatly reduced the sensitivity of the ninhydrin colour test for proline and hydroxyproline.

From here onwards the technique was essentially that of Campbell (1955). The samples were dissolved in water and 0·1–0·2 ml. samples pipetted on to 2 cm.² polythene planchets to which 10 μl. of 0·2% cetyltrimethylammonium bromide had been added. The samples were dried in a desiccator or at 60° and counted either for 5 min. or for 1000 counts, whichever gave the greater number of counts, in a Geiger end-window counter. After counting, the planchet contents were dissolved in water and the proline or hydroxyproline was estimated by the methods given above. The relation between counts/min. and μg. of imino acid present was linear up to at least 200 μg. The specific activities of proline and hydroxyproline samples measured before and after the complete isolation procedure agreed within 10%.

RESULTS

Free proline and hydroxyproline in the granuloma

A number of experiments were made to determine the nature of the free proline and hydroxyproline pools in the granuloma, in order to clarify the interpretation of the results of isotope-incorporation experiments. The free amino acids of the granuloma (about 18 mg. of trichloroacetic acid-soluble amino nitrogen/100 g. wet wt. of 8-days granuloma) were studied qualitatively by two-dimensional paper chromatography. The only unusual feature was the free hydroxyproline (1–2 mg./100 g. wet wt.), which was also present in the blood of guinea pigs carrying the granuloma (<0·5 mg./100 ml.). The free proline content was much larger (4–6 mg./100 g.) and of the same order as that of other amino acids present. The increase in proline and hydroxyproline in the slices and supernatant during a typical incubation is shown in Fig. 2. It

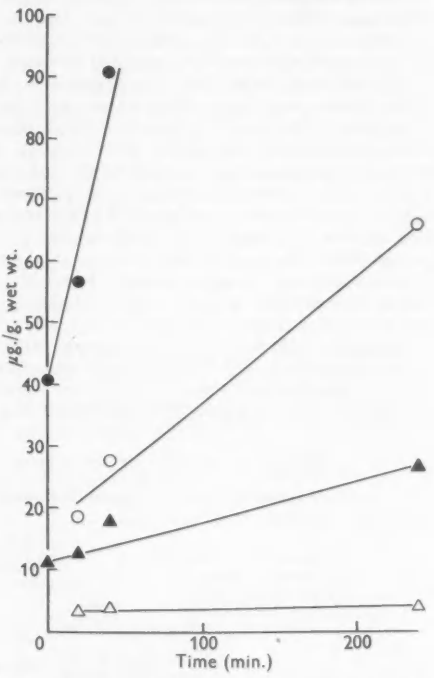

Fig. 2. Increase in free proline and hydroxyproline during incubation of tissue slices. ●, Total proline; ○, slice proline; ▲, total hydroxyproline; △, slice hydroxyproline. The 4 hr. point for total proline (300 μg./g. wet wt.) lay on the same line as the early points.

refer to the approximate amounts (Pro, proline; Hypro, hydroxyproline) isolated from each fraction for counting.

was much greater for proline than for hydroxyproline and in both cases it was directly proportional to incubation time. The increase in total amino nitrogen paralleled the increase in free proline, and paper chromatograms showed large increases in all the free amino acids present, suggesting that the proline arose from protein breakdown. The low hydroxyproline production shows that only a small proportion of this proline comes from collagen. Steinberg & Vaughan (1956) have observed similar increases in free amino acids during incubation of liver slices.

It was necessary to consider at some point the possibility that free hydroxyproline could not penetrate the fibroblast wall, since absence of its incorporation into collagen could be interpreted in terms either of a permeability barrier or of a pathway not involving free hydroxyproline. Although it seemed likely that hydroxyproline could penetrate into fibroblasts, since it was known to be taken up by ascites cells (Christensen, Riggs, Fischer & Palatine, 1952) and by intestinal mucosa (Wiseman, 1956), it was decided to test the point directly. Urea, raffinose, proline and hydroxyproline spaces were therefore measured on slices of 12-day-old granuloma, since this appeared to be more fibrous, with less extracellular space than 7-day tissue. The results, given in Table 1, show that proline and hydroxyproline were taken up by the cells of the granuloma to similar extents and were in fact concentrated within them. Unfortunately it is still possible that any lack of incorporation of free hydroxyproline could be due to a permeability barrier. The cell population of the granuloma is heterogeneous, so that although histological examination showed a predominance of fibroblasts it is possible, though unlikely, that all intracellular hydroxyproline is contained within other cells (muscle cells, macrophages and other white corpuscles) and that fibroblasts are impermeable to hydroxyproline. Furthermore, these experiments do not exclude an intracellular permeability barrier which could prevent access of exogenous hydroxyproline to the site of collagen synthesis, but which would not prevent utilization of endogenous hydroxyproline. These two possibilities are difficult to test experimentally and, since there is no independent support for them, they will be provisionally disregarded.

These experiments also showed negligible oxidative metabolism of proline or hydroxyproline during the 35 min. incubation period. This was confirmed in a number of other experiments on 6-day and 8-day tissue, which had been designed to detect direct oxidation of proline to hydroxyproline. No such conversion was observed with ordinary analytical techniques, and neither proline nor hydroxyproline was appreciably oxidized. Experiments with L-[^{14}C]proline showed that less than 0·1% of the radioactivity appeared in the free glutamic acid after incubation for 4 hr., showing that this pathway of proline breakdown is not of great importance in this tissue.

Specific activity of the proline pool

The change in specific activity of the proline of the supernatant amino acid and tissue amino acid fractions with time is shown in Table 2. The most remarkable feature of these results is the practically constant specific activity of the tissue amino acid proline. It changed very little between 10 min. and 4 hr., although the specific activity of the supernatant amino acid proline was two to four times as great throughout this period. Since the tissue amino acid fraction contains both extracellular and intracellular amino acids, the average specific activity of the intracellular proline pool may not have been constant, nevertheless it must have been much lower than either that of the extracellular proline or that of the total tissue amino acid proline. No reliable estimate of the specific activity of the intracellular proline could be made on

Table 1. *Distribution of* L-*proline and* L-*hydroxyproline between slices and medium*

Calculation of the results is described under Methods. The errors are standard deviations calculated from the results of four to six replicate incubations.

	Urea	Raffinose	Proline	Hydroxyproline
Space (expressed as % wet wt. of slices after incubation)	85·5±2	72±2	107±4	103±1
Intracellular concentration / Extracellular concentration	1	0	2·6±0·6	2·3±0·5

Table 2. *Specific activity of the proline pool*

Time of incubation (min.)	10	20	30	45	240
Supernatant amino acid (counts/min./μmole)	14 700	13 500	12 200	10 600	4 000
Tissue amino acid (counts/min./μmole)	2 700	2 100	2 600	2 900	2 000

account of the large proportion of extracellular space in the tissue, for even the washed slices retained over 50% by weight of medium, as measured by raffinose space.

Rate of uptake of [^{14}C]*proline into collagen*

After a lag period of 5 min. the specific activity of the neutral collagen proline increased linearly with time for at least 45 min. (Fig. 3). After 4 hr. the specific activity was about double that at 45 min. Calculation of the rate of collagen synthesis from Fig. 3 requires a knowledge of the specific activity of the intracellular proline pool and it will be considered later.

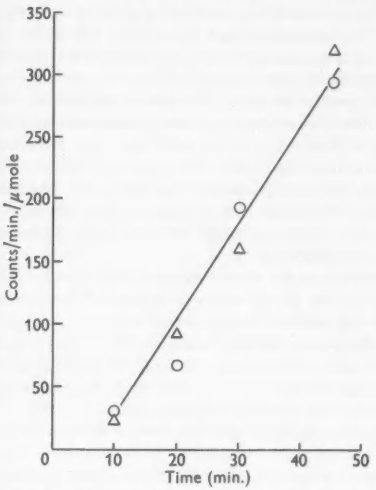

Fig. 3. Increase in specific activity of proline (○) and hydroxyproline (△) from neutral-salt-soluble collagen during incubation of tissue slices.

The specific activities of proline and hydroxyproline isolated from various collagen fractions after incubation for 4 hr. are shown in Tables 3 and 4. The relative activities of the neutral-salt-soluble, citrate-soluble and insoluble collagens are in accord with earlier experiments performed *in vivo* showing the uptake of [^{14}C]glycine into corresponding fractions from rabbit skin (Harkness *et al.* 1954) and from carrageenin-induced granuloma (Jackson, 1957). The total counts incorporated into neutral-salt-soluble collagen after 4 hr. were 20 times those in either citrate-soluble collagen or insoluble collagen. Since Jackson (1957) has shown that it is only the insoluble collagen which undergoes a large increase in total mass, the neutral collagen must be its main precursor.

The two water-soluble fractions were studied since it appeared possible that they might represent precursors of neutral-salt-soluble collagen. The fraction *A* probably contained any intracellular collagen in solution. It will be seen from Fig. 1 that the amount of hydroxyproline isolated was very small (15 μg. from 30 g. of slices). Fraction *B*, which corresponded to the 'water-soluble fraction' of Jackson (1957), contained considerably more hydroxyproline. Both fractions were quite highly labelled, although of lower specific activity than the neutral-salt-soluble collagen (Table 4). Unfortunately both were crude mixtures of proteins and contained only a small proportion of collagen, as can be seen from their relative contents of proline and hydroxyproline (Fig. 1). This difficulty was

Table 3. *Specific activities (counts/min./μmole) of proline and hydroxyproline from collagen fractions, after incubation for 4 hr.*

Specific activities in parentheses in the last column are based on low counts (<5/min.). The other insoluble-collagen specific activities are one-seventeenth of those obtained from a separate experiment in which 17 times as much [^{14}C]proline was added.

	Tissue amino acid	Neutral-salt-soluble collagen	Citrate-soluble collagen	Insoluble collagen
Hydroxyproline	—	1480	70	(4·6) 3·8
Proline	3900	910	50	(5·3) 3·9
$\frac{\text{Hydroxyproline}}{\text{Proline}}$	—	1·6±0·1 (average of six 4 hr. experiments)	1·3	(0·9) 1·0

Table 4. *Specific activities of hydroxyproline from the water-soluble fractions*

	Time of incubation (hr.)	Hydroxyproline (counts/min./μmole) From neutral-salt-soluble collagen	From water-soluble fractions *A*	From water-soluble fractions *B*
Expt. C. 28	0·5	144	79	—
Expt. C. 30	2	470	—	315

partly overcome by isolating hydroxyproline, which arises only from the collagen. However, it is likely that this hydroxyproline was metabolically heterogeneous and that it was derived both from denatured neutral-salt-soluble collagen (or possibly from a neutral-salt-soluble collagen precursor) and from degraded unlabelled collagen (gelatin) arising during incubation.

Conversion of proline into hydroxyproline

The specific activity of the neutral-salt-soluble collagen hydroxyproline was of the same order as that of the proline, showing that most of it must have been derived directly from proline. At early times (less than 40 min.) it was usually lower (the 20 min. point of Fig. 3 was the only exception), whereas later it was equal to or higher than the specific activity of the proline (Fig. 3 and Tables 3 and 5). If all the collagen hydroxyproline had been derived directly from proline (free or bound) without accumulation of intermediates, a constant specific-activity ratio of unity would have been expected, so that this variable ratio indicates some more complex process. It is especially difficult to understand how the collagen hydroxyproline could under any circumstances attain a specific activity higher than that of the proline. The earliest explanation considered was that it was an experimental artifact due to a protein impurity containing unlabelled proline. The neutral collagen was therefore purified further by three different methods, applied to different samples: (i) it was dissolved at pH 7·4 and precipitated with 15% NaCl; (ii) it was redissolved at pH 3·8 and precipitated with 5% NaCl; (iii) it was redissolved at pH 7·4 and the fraction taken which underwent reversible gelling when warmed to 37° (Fessler, 1956). None of these procedures affected the hydroxyproline/proline specific-activity ratio or the hydroxyproline/nitrogen ratio. Further discussion of this problem is deferred until later.

The ratios of the specific activity of hydroxyproline to that of proline from the citrate-soluble and insoluble collagen fractions was lower than the corresponding ratio for the neutral-salt-soluble fraction (Table 3). This is possibly because much of the radioactivity in these fractions passed through the neutral-salt-soluble collagen stage at relatively early times during the incubation when the ratio was lower than it was after 4 hr.

In order to test for free hydroxyproline as an intermediate in the formation of collagen hydroxyproline, unlabelled L-hydroxyproline was added to the incubation mixture (Table 5). At mM concentration it had little effect, but at 10 mM the specific activities of both proline and hydroxyproline were considerably reduced. There was apparently some inhibition of collagen synthesis without any dilution of hydroxyproline radioactivity relative to proline, showing that free hydroxyproline could not be an intermediate. Inhibition by hydroxyproline of incorporation of proline into proteins has also been observed by Steward, Pollard, Patchett & Witkop (1958).

A study of the radioactivity of the free hydroxyproline from the tissue and supernatant amino acid fractions leads to similar conclusions about its role in collagen synthesis (Table 5). There was no great difference between specific activities of the two fractions, which were both less than half that of the neutral-salt-soluble collagen hydroxyproline. In itself this specific activity was not so low as to exclude free hydroxyproline from the role of intermediate, since the pool might have been metabolically heterogeneous. However, addition of unlabelled hydroxyproline did not trap any additional counts in this fraction, whose specific activity therefore fell sharply. It seems likely that this labelled free hydroxyproline arises from the irreversible breakdown either of labelled collagen or of some hydroxyproline-containing precursor. After these experiments were completed it was

Table 5. *Effect of adding unlabelled hydroxyproline* (1) *on the incorporation of* L-[^{14}C]*proline into collagen and* (2) *on the specific activity of free hydroxyproline*

Specific activities have been multiplied by the factor 4000/specific activity of tissue amino acid proline, in order to correct for differences in the specific activity of the amino acid pool. Figures in parentheses are total counts isolated.

		Specific activities of proline and hydroxyproline (counts/min./μmole)				
		Neutral-salt-soluble collagen				
Incubation time (hr.)	Concn. of hydroxyproline added (mM)	Hydroxyproline	Proline	Hydroxyproline / Proline	Tissue amino acid Hydroxyproline	Supernatant amino acid Hydroxyproline
2	0	780	680	1·15	—	88 (22)
2	10	440	400	1·10	—	0·6 (36)*
4	0	1510	930	1·64	370 (27)	380 (73)
4	1	1450	860	1·69	—	—
4	10	830	520	1·60	3·4 (20)*	1·6 (88)*

* Values are based on low counts (<5/min.) and therefore approximate.

Table 6. *Comparison of* [^{14}C]*proline and* [^{14}C]*hydroxyproline as sources of neutral-salt-soluble collagen hydroxyproline*

Labelled amino acid added	Incubation time (hr.)	Activity added (counts/min.)	Counts/min./μmole: Supernatant amino acid: Proline	Supernatant amino acid: Hydroxyproline	Tissue amino acid: Proline	Tissue amino acid: Hydroxyproline	Neutral-salt-soluble collagen hydroxyproline
Proline	0	150 000	—	—	—	—	2·1
Proline	2	150 000	5200	—	2400	—	465
Hydroxyproline	2	27 000	—	2900	—	3500	2·3

found that the labelled proline used contained traces (0·03 %) of labelled hydroxyproline, which could be isolated from it by adding carrier, and treating with nitrous acid and chromatographing several times in butanol–acetic acid–water. This hydroxyproline accounted for a considerable proportion of the counts in the free hydroxyproline fractions. However, the 2 hr. experiment (Table 5), for which L-[^{14}C]proline purified by butanol–acetic acid–water chromatography was used, shows that some of the radioactivity was genuinely derived from proline. The presence of this labelled hydroxyproline does not therefore affect the conclusions drawn from these experiments.

To obtain final confirmation that free hydroxyproline could not be incorporated into collagen, labelled hydroxyproline was incubated with tissue slices in the usual system and the specific activity of the amino acid and neutral-salt-soluble collagen fractions were determined (Table 6). The incorporation of labelled hydroxyproline was negligible. The 2 counts/min. which did appear were quite possibly due to slight contamination of the neutral collagen by labelled free hydroxyproline, since in a control experiment similar activity appeared in proline when neutral-salt-soluble collagen was isolated in the presence of labelled proline added after incubation.

DISCUSSION

Collagen precursors

Some form of soluble collagen is now firmly established as the precursor of the collagen fibre. Orekhovitch & Shpikiter (1957) maintain that citrate-soluble collagen (so-called procollagen) is the precursor, although Harkness *et al.* (1954) and Jackson (1957) have shown quite clearly that the neutral-salt-soluble fraction becomes labelled both more rapidly and more highly than the citrate-soluble fraction. Moreover, Jackson (1957) has pointed out that the method used by Orekhovitch to extract citrate-soluble collagen will also extract neutral-salt-soluble collagen, so that his 'procollagen' is a mixture of the two fractions. Our results confirm that the neutral-salt-soluble collagen fraction represents the earliest stages of collagen synthesis, but before making categorical statements about a particular fraction being a collagen precursor the fractionation procedure should be examined in more detail.

Superficially, neutral-salt-soluble and citrate-soluble collagens appear to be metabolically distinct fractions, but it is becoming increasingly apparent that this is misleading. It seems more accurate to regard them as arbitrary fractions from a continuous series of 'collagens' of decreasing solubility. Once the collagen is in solution its properties are largely independent of the method of extraction, provided that there has been no denaturation. The amino acid compositions of the neutral and citrate-soluble fractions are almost the same (Jackson, Leach & Jacobs, 1958) and there is increasing evidence that the two fractions cannot be distinguished on physicochemical grounds (Peng & Tsao, 1956; Orekhovitch & Shpikiter, 1957; Bensusan & Hoyt, 1958). Both Gross (1956, 1958*b*) and Bensusan & Hoyt (1958) have shown that when acid-soluble collagen is dialysed against neutral buffers it remains in solution, provided that the ionic strength is greater than 0·15. The resulting neutral solutions have the same fibre-forming properties as solutions of neutral-salt-soluble collagen. The arbitrary nature of the distinction is further emphasized by the experiments of Gross (1958*a*), who found that 0·45 M-sodium chloride solution extracted five times as much collagen from skin of young guinea pigs as did 0·2 M-sodium chloride solution.

The continuous gradation of solubilities of the constituents of the collagen fibre has a metabolic parallel in continuously decreasing specific activities of the collagen from consecutive citrate extracts of rabbit skin, corresponding to less and less soluble fractions (Harkness *et al.* 1954; Orekhovitch & Shpikiter, 1957). We have repeated this observation on successive neutral salt extracts of the granuloma. It is therefore incorrect to refer to a collagen fraction isolated by a particular extraction method as the precursor of the collagen fibre since all the solvents used extract more or less fibrous collagen in addition to collagen in solution. The amount of collagen actually present in solution *in vivo* at any one time is very small and presumably appears in the first extract in whatever

solvent is being used, along with varying amounts of collagen derived from the fibre. The 0·2M-neutral-salt-soluble fraction contains only the most recently laid down and hence most highly labelled collagen whereas the citrate extract includes more older material formed before the introduction of the labelled amino acid. The 0·45M-sodium chloride solution extract of Gross (1958*a*) may occupy an intermediate position.

Careful observation of electron micrographs of collagen fibres led Gross, Highberger & Schmitt (1954) to postulate the existence of an elongated collagen particle or molecule (tropocollagen), probably secreted by the fibroblast and subsequently aggregating more or less spontaneously to form collagen fibres (Gross, 1956). The existence of such particles in collagen solutions has been amply confirmed by Boedtker & Doty (1956). On the basis of the evidence from incorporation of isotopic amino acids it has usually been assumed that the neutral-salt-soluble collagen fraction contains the tropocollagen of Gross *et al.* (1954). However, there has been no evidence to exclude a soluble precursor representing an even earlier stage of collagen formation than the neutral-salt extract. Jackson (1957) commented on the bound hydroxyproline content of water-soluble fraction *B* and suggested that it might contain a neutral-salt-soluble collagen precursor. Our measurement of the specific activity of the hydroxyproline of the two water-soluble fractions *A* and *B* (Table 4) show that this can be so only if these fractions contain a high proportion of metabolically inert collagen in addition to the hypothetical precursor. Since no purification was attempted, this is not impossible. However, evidence has recently been obtained which locates more precisely the intracellular source of neutral-salt-soluble collagen. It has been found (N. M. Green & D. A. Lowther, unpublished work) that bound hydroxyproline isolated from the 'microsome fraction' after incubation of granuloma slices with labelled proline has nearly four times the specific activity of the neutral-salt-soluble collagen hydroxyproline. Moreover, this hydroxyproline can be extracted from the microsomes with 0·14M-sodium chloride solution and coprecipitated with carrier neutral-salt-soluble collagen, providing good evidence for the chemical identity of collagen extracted from the fibres by neutral-salt solutions with microsomal collagen, which is probably the first collagen to be formed.

Conversion of proline into hydroxyproline

The results presented in Table 6 show that free hydroxyproline is not incorporated into the neutral-salt-soluble collagen of granuloma slices whereas under the same conditions both proline and glycine (unpublished experiments) are incorporated. Moreover, both the proline and the hydroxyproline of the soluble collagens isolated from slices incubated with L-[^{14}C]proline are approximately equally labelled and the addition of hydroxyproline does not alter their relative specific activities (Table 5). These experiments suggest that collagen hydroxyproline is derived from added proline, as previously suggested by Stetten (1949), and that free hydroxyproline is not an intermediate. Stetten's results have recently been confirmed by Jackson & Smith (1957), who studied the incorporation of [^{14}C]proline into the protein of osteoblasts *in vitro*, by Mitoma & Smith (1957) who used a granulation-tissue mince and by Wolf & Berger (1958) who fed L-[^{14}C]hydroxyproline to rats. The most likely explanation of these results is that proline is hydroxylated only in a bound form (Stetten, 1949) and that the resulting bound hydroxyproline is incorporated directly into collagen. The alternative mentioned above, that there is an intracellular permeability barrier preventing access of exogenous hydroxyproline to the site of collagen synthesis, is difficult to test experimentally and will be provisionally disregarded. Although our results do not allow any direct inferences to be made about the nature of the bound proline which is hydroxylated, the ratio of specific activities of hydroxyproline/proline provokes a number of questions related to this problem. This ratio increases with time from a value of 0·5–0·7 after 10 min. to 1·0 at about 40 min. and to 1·6 after 4 hr. (Fig. 3 and Table 3). Ratios greater than 1 were also obtained *in vivo*, when collagen was isolated from rat skin after feeding L-[^{14}C]proline (A. Neuberger & F. Charconnet, unpublished work). We first considered that the high ratio might be due to a protein impurity containing proline of low specific activity, but further purification of neutral-salt-soluble collagen was without effect. Furthermore, the amounts of such an impurity required would be so large that the amino acid composition of the collagen would be changed. Since neutral-salt-soluble collagen from rabbit skin has almost the same amino acid composition as the other collagen fractions (Jackson *et al.* 1958), and since our neutral-salt-soluble fraction has a normal hydroxyproline content (7·7 g./100 g. of protein nitrogen) this explanation is untenable. Another possibility is that there is a source of unlabelled proline which can enter collagen without mixing completely with the pool of labelled free proline, thus continually diluting collagen proline relative to hydroxyproline. The breakdown of cell proteins could fulfil this role provided that there was some direct coupling of breakdown to collagen synthesis. This could take the form either of an activated proline derived from protein breakdown or of a physical inhomogeneity in the intracellular amino

acid pool whereby amino acids from protein breakdown were preferentially reutilized for synthesis of collagen or other protein. The low specific activity of hydroxyproline relative to proline at early times requires a separate explanation. It is possible that there is a larger accumulation of intermediates between free proline and collagen hydroxyproline than between free proline and collagen proline. Approximate calculations show that an extra pool equivalent to about 1–2 μg. of hydroxyproline/g. of tissue on the hydroxyproline pathway could account for the observed results (Fig. 3). The last two hypotheses are incompatible with hydroxylation of proline after completion of the collagen peptide chain. In general it can be stated that hydroxylation at this stage cannot be reconciled with the hydroxyproline/proline specific-activity ratios without making even more complicated assumptions.

Rate of synthesis of collagen

In order to calculate the rate of turnover of neutral-salt-soluble collagen from the isotope-incorporation data it is necessary to know the specific activity of the amino acid pool from which it was formed. Unfortunately it is impossible to obtain an accurate estimate of this from our data. The tissue amino acid fraction isolated from the washed slices contained a considerable proportion of extracellular amino acid, whose specific activity was presumably close to that of the proline in the incubation medium. This would not matter if there had not been so high a ratio of the specific activity of the proline of the supernatant amino acid fraction to that of the tissue amino acid fraction. This ratio, which fell only slowly during the experiment in spite of the high specific-activity gradient, was probably of the same origin as that observed in liver *in vivo* (Loftfield & Harris, 1956) and in perfused lung (Askonas & Humphrey, 1958), although its magnitude was initially about double that found in these systems. Either there was a pool of free intracellular proline which remained unlabelled and which exchanged only slowly with the pool from which collagen was synthesized, or the continuous turnover of intracellular protein maintained the specific activity of the intracellular proline below that in the incubation medium. The second hypothesis seems the more likely on general grounds, moreover the existence of a high protein turnover receives support from the large amounts of proline liberated from the slices during incubation. It is interesting to contrast the behaviour of hydroxyproline, whose specific activity was almost the same in both supernatant and tissue amino acid fractions (Tables 5 and 6) and which was liberated from the slices at only one-fifteenth of the rate of proline liberation. Unfortunately, this could be explained equally well in terms of either hypothesis.

In view of the uncertainties attached to any estimate of the specific activity of the amino acid pool it is perhaps more informative to assume a maximum rate of synthesis equal to that found by Jackson (1957) *in vivo* and to calculate from this a minimum specific activity for the pool proline. For a 7-day granuloma Jackson found that 6 μg. of collagen hydroxyproline/g./hr. was synthesized, whereas the total neutral-salt-soluble collagen did not change significantly. Since the pool of neutral-salt-soluble collagen hydroxyproline was 40 μg./g. of slices in our experiments, this rate corresponds to 15% replacement/hr. The average specific activity of the free proline pool should therefore be approximately 100/15 times that of the neutral-salt-soluble collagen proline after incubation for 1 hr., since only 5% of the counts in the neutral-salt-soluble collagen were transferred to the acid-soluble and insoluble collagen fractions. From consideration of a 30 min. period during the linear portion of Fig. 3, the average specific activity of the proline pool would be

$$\frac{100 \times 235}{7{\cdot}5} = 3100 \text{ counts/min./}\mu\text{mole.}$$

This is slightly higher than the specific activity of tissue amino acid proline during this period (Table 3). Since this fraction contained considerable amounts of extracellular proline of high specific activity the average specific activity of the intracellular proline was lower than that of the whole tissue amino acid. It is therefore difficult to account for the higher specific activity just calculated, unless the intracellular pool is heterogeneous. Although this is not unlikely, there is insufficient evidence for further discussion.

SUMMARY

1. When slices of granuloma induced by carrageenin were incubated in the presence of L-[^{14}C]proline, both proline and hydroxyproline isolated from the soluble and insoluble collagen fractions were labelled. After a lag period of 5 min. the specific activities of proline and hydroxyproline from neutral-salt-soluble collagen increased linearly with time for at least 45 min. After 4 hr. the total counts in this fraction were 20 times those in the acid-soluble or insoluble collagen fractions, confirming that neutral-salt-soluble collagen represents an early stage in collagen-fibre formation.

2. The specific activity of hydroxyproline from neutral-salt-soluble collagen increased from approximately 0·6 to 1·6 times that of the proline during the incubation for 4 hr. Tentative explanations for this variable ratio were put forward and it

was pointed out that it would be difficult to reconcile with any scheme of collagen synthesis involving the hydroxylation of proline as a final stage.

3. The addition of unlabelled L-hydroxyproline to the incubation medium in the presence of L-[^{14}C]proline had no effect on the ratio of the specific activities of collagen hydroxyproline and proline although the total radioactivity incorporated was reduced.

4. Incubation of the tissue with L-[^{14}C]hydroxyproline did not result in a significant incorporation of radioactivity into collagen.

5. Radioactive free hydroxyproline was isolated from the slices and medium after incubation with L-[^{14}C]proline but its specific activity was only half that of the neutral-salt-soluble collagen hydroxyproline and the total counts present were not increased when unlabelled hydroxyproline was present as a trapping agent.

6. It is concluded that free hydroxyproline is not an intermediate in the formation of the hydroxyproline of collagen.

7. Both proline and hydroxyproline added to the medium were found to be concentrated intracellularly about 2·5 times. The lack of incorporation of free hydroxyproline cannot therefore be due to the impermeability of the cells towards hydroxyproline.

8. The specific activity of the free proline from the slices increased to 20 % of that in the incubation medium within 10 min. but no further increase occurred in spite of the high specific activity of the proline from the medium. Although the latter fell to approximately one-third of its initial value during incubation for 4 hr., owing to dilution by proline liberated from the slices, it was never less than twice the specific activity of the slice proline.

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Studies on the Preparation and Isoelectric Point of Staphylococcal α-Haemolysin

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Studies on the physicochemical properties of staphylococcal haemolysin have been hampered by the difficulty of preparing a pure, active product in sufficient quantity. Wittler & Pillemer (1948) described a three-step purification procedure which gave a product of high activity, but the results were not entirely reproducible and they did not pursue the study (L. Pillemer, personal communication). More recently, Turpin, Relyveld, Pillet & Raynaud (1954) described a four-step procedure which resulted in a material of high toxicity. Even this material was still a complex mixture, however, as shown by serological analysis. Both these methods were tried but gave unsatisfactory results when filtrates of other strains were used. The work described here was an attempt to fractionate α-toxin preparations by the use of ammonium sulphate, ethanol and Cellosolve. The isoelectric point was measured on toxin which had been further separated by electrophoresis.

MATERIALS AND METHODS

Filtrates

A strain of *Staphylococcus aureus*, designated A99 and isolated by Dr E. Levy from a human lesion, was grown in a modified Walbum medium. This consisted of Difco heart-infusion broth to which 0·2% of KH_2PO_4 and 0·03% of $MgSO_4,7H_2O$ were added. The solution was then treated with 1% (w/v) of activated charcoal for about 1 hr., filtered and the pH adjusted to 7·4 with NaOH. It was dispensed in 100 ml. quantities in 16 oz. flat bottles, and each bottle was inoculated with 0·5 ml. of an overnight broth culture of A99. The cultures were incubated at 37° in a mixture of CO_2+air or oxygen (30:70), for 68 hr. in a horizontal position. The bottles were gently rocked a few times daily. The cells were removed by centrifuging in a Sharples centrifuge, and Merthiolate (thiomersal) was added to the filtrate to a concentration of 0·01%.

A filtrate was also obtained from the Wellcome Research Laboratories, Beckenham, Kent. This was derived from the Wood 46 strain grown in a medium containing horse-muscle infusion.

Fractionation procedures

Ammonium sulphate. The pH of the filtrate was adjusted to 6·5 (glass electrode) by the addition of dil. HCl or dil. acetic acid. The solution was then cooled in ice and saturated with $(NH_4)_2SO_4$. After standing overnight at 0°, the precipitate was centrifuged at 10 000 rev./min. in a Spinco model L ultracentrifuge at 0°. The precipitate from each 2·5 l. of filtrate was extracted in about 130 ml. of water. The residue was centrifuged down and discarded. The extract was dialysed at 0°, first against tap water and finally against distilled water, until free of $(NH_4)_2SO_4$ (usually about 3–4 days when dialysed into about 50 times its volume, changed two or three times each day). Any precipitate obtained by dialysis was discarded and the solution freeze-dried. Variations in this method, consisting of changes in the concentration of $(NH_4)_2SO_4$ at different values of pH, will be referred to later.

Ethanol. Ethanol fractionations were carried out on preparations obtained from precipitations with $(NH_4)_2SO_4$. A 1% (w/v) solution of the precipitate was prepared in sodium acetate, *I* 0·05, pH 7·2. The pH of the solution was 6·9. It was cooled to 0°, and the required volume of ethanol, previously cooled to −17°, was added slowly with gentle but efficient stirring, and the mixture allowed to stand at −17° overnight. The precipitate was then centrifuged down at −5°, dissolved in water, and dialysed in the same way as the $(NH_4)_2SO_4$ preparation and freeze-dried; the supernatant was cooled to −17° in preparation for further fractionation with more ethanol. In this way precipitates were obtained with 0·5, 1·0, 1·5, 2·0, 4·0 and 6·0 vol. of ethanol.

Cellosolve. Cellosolve (2-methoxyethanol) fractionation was also carried out on a solution of an $(NH_4)_2SO_4$ preparation. A 1% (w/v) solution of the preparation in 0·15 M-sodium acetate was prepared, and cooled to 0° and 1 volume of Cellosolve, previously cooled to −17°, was added slowly with gentle but efficient stirring. The mixture was stood at −17° for 6 hr. and then centrifuged at −10° to obtain the precipitate. The supernatant was cooled to −17° and a further volume of cold Cellosolve added and treated as before. The precipitates were dissolved in 0·15 M-sodium acetate and a portion of each solution was kept at 4°, whilst the remainder was dialysed in the same way as the $(NH_4)_2SO_4$ preparation and freeze-dried.

Analytical procedures

Determination of haemolytic activity. The haemolytic activity against rabbit red blood cells was determined by the method of doubling dilution in tubes, the first tube containing a dilution of 1:10. Care was taken to cause only minimal frothing when the dilutions were mixed. An equal volume of a 2% (w/v) suspension of washed rabbit red blood cells was added to each tube and the tubes were shaken and incubated for 1 hr. in a water bath at 37°. Those showing partial haemolysis were centrifuged at about 1500 rev./min. for 5 min. and the supernatants compared visually with a standard showing 50% lysis. An

estimate was then made of the final toxin dilution which would have caused 50% haemolysis. The reciprocal of the dilution was taken to be the number of haemolytic units/ml. of the undiluted toxin solution. Whenever possible, the red blood cells were collected from the same rabbit in order to overcome variations in red-cell susceptibility. When another rabbit had to be used, the new cells were compared with those from the original. The cells were washed in 0·15M-NaCl before use and stored in the packed state at 4°. They were discarded after 1 week.

Determination of lethal activity. The lethal activity was determined by injecting 0·5 ml. portions of appropriate dilutions of toxin, dissolved in 0·15M-NaCl, intravenously into Swiss white mice of approx. 20 g. weight. After 24 hr. the LD_{50} was recorded. Two mice were used for each dilution for economy and it was found that they gave sufficiently reliable results.

Agar-diffusion analysis. A modification of the Elek (1948, 1949) double-diffusion-gradient method was used. Rectangular ditches (2 cm. × 0·5 cm.) were cut at right angles to each other about 0·5 cm. apart in a plate poured with filtered 2% (w/v) Noble (Difco) agar in 0·15M-NaCl. Solutions of the toxin preparations (1%, w/v) were mixed with equal volumes of the molten-agar solution previously cooled to 56° and pipetted into one set of ditches. The other set of ditches received a 1:1 mixture of staphylococcal antiserum (Burroughs Wellcome and Co.) and agar. The plates were exposed to formaldehyde vapour and left at room temperature until the antigen–antibody lines developed.

Nitrogen determinations. Nitrogen determinations were carried out with a micro-Kjeldahl technique with hydrogen peroxide as the catalyst.

Paper electrophoresis. Paper electrophoresis was carried out in a horizontal apparatus (Franglen, 1953). Sheets of Whatman no. 1 filter paper, 24 cm. wide, were used at a voltage of 4 v/cm. for 24 hr. Sodium acetate–acetic acid, Na_2HPO_4–KH_2PO_4 and sodium barbitone–diethylbarbituric acid buffers, all at I 0·1, gave average currents of 1·1, 1·3 and 1·95 mA respectively. The toxin preparations were applied as 4% (w/v) solutions and run in quadruplicate. Dextran (6%, w/v) was applied between the toxin fractions to indicate the endosmotic flow. All runs were performed in the cold room at 4°. At the end of the run, the sheet was cut into strips and two were placed on a large plate previously poured with saline–agar containing washed rabbit red blood cells. The plate was incubated at 37° in order to determine the position of the haemolytic factor. After incubation for 45 min. the strips were removed and the plate was inspected and photographed without delay. It was found that with longer incubation diffusion occurred, which obscured the exact position of the haemolytic factor. The paper strip after removal was found to be stained with haemoglobin at the site of the haemolytic effect, but this could not be utilized as a record because of diffusion. The remaining strips were stained for protein or carbohydrate by means of bromocresol green and periodic acid–Schiff reagent respectively.

Tiselius electrophoresis. The apparatus used was described by Butler, James & Treherne (1957). Solutions of toxin (1%, w/v) dissolved in either Na_2HPO_4–KH_2PO_4 buffer, pH 6·0, 6·9, 7·46 and 8·0, or sodium barbitone–diethylbarbituric acid buffer, pH 8·7, all at I 0·075, were used. Electrophoresis was carried out with a current of 5 mA for 2–4 hr. depending on pH. Fractions were removed from the electrophoretic cell by the method of Butler & Stewart (1957). Briefly, this consists in removing very small portions from the limb of the electrophoretic cell by means of a fine needle, which is finely controlled mechanically to avoid disturbance in the fluid. The resistance of the buffer solutions was determined at 0° in a conductivity cell at 1500 cycles/sec.

RESULTS

Fractionation with haemolytic activity as the indicator. Holt (1936) precipitated staphylococcal α-toxoid by full-saturation with ammonium sulphate at pH 6·5. He mentioned that much toxin also precipitated at half-saturation. Studies were therefore made of the effect of various concentrations of ammonium sulphate at varying pH. A product prepared by precipitation with full saturation with ammonium sulphate was freeze-dried. A 1% (w/v) solution of this was made, divided into six parts and cooled in ice. A range of pH from 4·2 to 6·6 was obtained by adjusting with dilute acetic acid, with a glass electrode. Precipitates appeared which were centrifuged off in weighed centrifuge tubes. Ammonium sulphate, to give 0·25 saturation, was added slowly at 0° and the fraction collected in weighed centrifuge tubes after standing

Table 1. *Weights and haemolytic activities of fractions obtained by fractional precipitation with ammonium sulphate at varying* pH *values*

Solutions (1%, w/v) of a freeze-dried preparation were obtained by full saturation of staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5; pH was adjusted with dil. acetic acid. Precipitations were carried out at 0°. Initial weights, at pH 4·20, 4·50, 5·05, 5·36 and 6·61, were 100 mg.; at pH 5·94, 60 mg. Weights are expressed in milligrams, and activities in haemolytic units/mg.

Degree of saturation with $(NH_4)_2SO_4$	pH 4·20		pH 4·50		pH 5·05		pH 5·36		pH 5·94		pH 6·61	
	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity
0	6·4	20	5·2	365	2·7	93	2·8	457	2·0	640	1·4	114
0·25	9·5	34	6·5	25	4·3	30	4·9	65	2·7	59	3·9	33
0·50	32·5	2400	31·2	3200	30·7	400	25·0	300	5·0	—	20·8	400
0·75	20·6	1200	27·9	1600	18·4	1600	29·5	1600	11·4	2000	19·8	1600
1·0	5·6	150	11·9	300	7·1	2500	6·4	600	5·8	500	18·0	3200

overnight. The supernatant was then treated with more ammonium sulphate and the procedure repeated. Each precipitate was dissolved in water, dialysed until free of ammonium sulphate, and freeze-dried and its haemolytic activity determined. The results are summarized in Table 1, which shows that less ammonium sulphate is required to precipitate the haemolysin at a lower pH value than at a higher: for example, 0·5 saturation at pH 4·5 gave a product of activity comparable with full saturation at pH 6·6. However, for the production of further batches, it was decided to keep to full saturation at pH 6·5 as exposure to low pH might be undesirable.

Double-diffusion plates (shown diagrammatically in Fig. 1) showed that the product obtained by saturation with ammonium sulphate consisted of at least five components, and the electrophoretic patterns were also complex (Fig. 2). Fractional reprecipitation with ammonium sulphate failed to result in increased activity, or to reduce the complexity of the double-diffusion pattern.

Ethanol fractionation of a solution of the product obtained by full saturation with ammonium sulphate gave less complex preparations, as judged by double diffusion. However, they had lower activity, and in some cases had no activity at all. The results of a typical fractionation are given in Table 2, and the diffusion patterns are shown diagrammatically in Fig. 1. Both the 1·0-vol. and the 1·5-vol. precipitates shared two lines with the parent substance, but the 1·0-vol. precipitate, which was haemolytic, had two lines unshared with the inactive 1·5-vol. precipitate. One of these might represent the haemolysin.

Fractionation with Cellosolve also gave products which were less complex, as shown by the double-

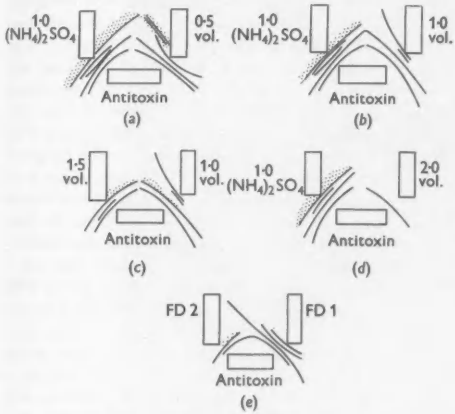

Fig. 1. Diagram of agar double-diffusion pattern obtained with fractions precipitated by ammonium sulphate, ethanol and Cellosolve. (*a*) Full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 0·5 vol. of ethanol (0·5 vol.); (*b*) full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 1·0 vol. of ethanol (1·0 vol.); (*c*) 1·5 vol. of ethanol (1·5 vol.) and 1·0 vol. of ethanol (1·0 vol.); (*d*) full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 2·0 vol. of ethanol (2·0 vol.); (*e*) 1 vol. of Cellosolve, freeze-dried (FD 1) and 2·0 vol. of Cellosolve, freeze-dried (FD 2). —, Line of antigen–antibody precipitation; ⁞⁞⁞⁞, areas of diffuse precipitation; □, antigen and antiserum ditches.

Table 2. *Haemolytic and lethal activities of fractions obtained by fractionation with ethanol*

Fractions were obtained by adding the required volume of ethanol, previously cooled to −17°, to a cold 1% (w/v) solution of a freeze-dried preparation obtained by full saturation of staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5, dissolved in sodium acetate, *I* 0·05.

Fraction (vol. of ethanol)	Haemolytic activity: Units/mg.	Haemolytic activity: Units/mg. of N	LD_{50} (mg.)
'Parent'	150	1028	0·05
0·5 vol.	75	—	—
1·0 vol.	88	620	0·10
1·5 vol.	—	—	>0·2
2·0 vol. 4·0 vol. 6·0 vol.	None		

Table 3. *Haemolytic and lethal activities of fractions obtained by fractionation with Cellosolve*

Fractions were obtained by adding the required volume of Cellosolve, previously cooled to −17°, to a cold 1% (w/v) solution of a freeze-dried preparation obtained by full saturation of a staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5, and dissolved in 0·15M-sodium acetate.

Fraction and vol. of Cellosolve	Haemolytic activity: Units/mg.	Haemolytic activity: Units/mg. of N	LD_{50}
'Parent'	200	1263	0·02 mg.
1·0 vol. (soln.*)	—	2700	0·0075 ml.
1·0 vol. (FD 1†)	800	6400	0·009 mg.
2·0 vol. (soln.*)	—	1173	0·09 ml.
2·0 vol. (FD 2†)	38	262	>0·3 mg.

* Precipitate was dissolved in 0·15M-sodium acetate.
† Precipitate was dissolved in 0·15M-sodium acetate, dialysed and freeze-dried.

diffusion technique and, in contrast with ethanol, gave a preparation with increased activity compared with the parent substance. Table 3 shows the biological activities of the substances obtained. It appears that Cellosolve is less harmful to the toxin than ethanol but it is still not selective enough to separate the toxin from the other protein material present.

Ratios of lethal/haemolytic activity. The L/H (lethal/haemolytic activity) ratios were obtained by measuring the two activities of each substance at the same time with portions taken from the same solutions. Results for the various products are shown in Table 4, and to some extent the effect of the various reagents on the biological activities can be seen. Reprecipitation with ammonium sulphate of the product originally obtained by precipitation with ammonium sulphate did not alter the ratio, but the original precipitation with ammonium sulphate and further fractionations with ethanol or Cellosolve caused marked changes. Dialysis and freeze-drying of the Cellosolve fractions also caused the ratios to increase. These results indicate that the two activities were being affected differentially by the procedures used.

Paper electrophoresis of the haemolytic factor. Although the toxin preparations were by no means pure, it was hoped that the haemolytic factor could be identified by electrophoresis and perhaps even concentrated sufficiently to enable further purification. Paper electrophoresis was used as a screening method in order to conserve material. The relationship between pH and the distance migrated (corrected for the endosmotic flow) by the haemolytic factor indicated that the haemolytic factor has an isoelectric point below pH 4·5. Strips stained for protein were very weakly coloured and no satisfactory measurements could be made. The strips did not show any polysaccharide to be present.

Tiselius electrophoresis of the haemolytic factor. When material was examined by electrophoresis in a Tiselius apparatus, followed by the determination of the haemolytic activities of the fractions removed from the cell, a different value was obtained for the isoelectric point. The schlieren pattern was complex. Some fast-moving components appeared first, which were shown to have no haemolytic activity and in subsequent runs were allowed to migrate out of the limbs. The bulk of the material stayed near the origin, but when the ionic strength of the buffer used was lowered from 0·2 to 0·075, this large peak began to split. The position of the peaks obtained at various pH values and the limits of significant haemolysis could not be correlated, and therefore it was concluded that the active factor was present in amounts too small to be detectable by the method. The instrument is capable of showing a peak at about 0·02 % of protein, so that the active factor must have been present at concentrations less than this. It was therefore decided to use the electrophoretic pattern obtained at any pH only as a check between comparable runs, particularly with different batches of equivalent preparations, as the main peaks always behaved in the same way. Fractions were then removed from the same levels in duplicate experiments. Owing to the design of the Tiselius cell, no band of activity is obtained as in zone electrophoresis, but instead the limit of migration is observed. All the experiments were so arranged that migration of the active substance occurred into the pure buffer, since the protein side of the original boundary was always active. In the figures shown, the buffer side of the boundary is on the left.

Table 4. *Comparison of lethal/haemolytic activity (L/H) ratios*

Measurements of haemolytic and lethal activities of the staphylococcal filtrates and of the various preparations obtained from them were carried out at the same time with portions taken from the same solutions.

Parent substance	Derivative	L/H ratio
1·0 sat. $(NH_4)_2SO_4$, 12/12/55	—	13·3
	0·75 sat. $(NH_4)_2SO_4$ ppt.	13·3
	1·0 sat. $(NH_4)_2SO_4$ ppt.	13·3
Filtrate, 20/4/56		5
	1·0 sat. $(NH_4)_2SO_4$ ppt.	8
Filtrate, 20/8/56		1·3
	1·0 sat. $(NH_4)_2SO_4$ ppt.	5
Filtrate, 21/9/56		2·8
	1·0 sat. $(NH_4)_2SO_4$ ppt.	6·6
1·0 sat., 20/8/56 21/9/56 0·75 sat. $(NH_4)_2SO_4$ ppt.		4·0
	1 vol. Cellosolve ppt. (soln.)	5
	2 vol. Cellosolve ppt. (soln.)	10
	1 vol. Cellosolve ppt. (freeze-dried)	7
	2 vol. Cellosolve ppt. (freeze-dried)	(not lethal)
1·0 sat. $(NH_4)_2SO_4$, 9/11/56		3
	1 vol. of ethanol	10

The schlieren patterns obtained by electrophoresis in phosphate buffer, I 0·075, at pH 6·0, 6·9 and 8·0, are shown in Fig. 2, together with the haemolytic activities of the fractions removed. If the fraction with 25% of the original starting activity is taken as indicating the position of the haemolytic factor, and the values of the mobilities of these fractions are plotted against pH, the position of the isoelectric point can be obtained. Fig. 3 shows the graph so obtained and the isoelectric point occurs at pH 6·4. The patterns obtained by electrophoresis at pH 6·95 in the descending limb for 261 min. are shown in Fig. 4. No activity migrating with a positive charge was

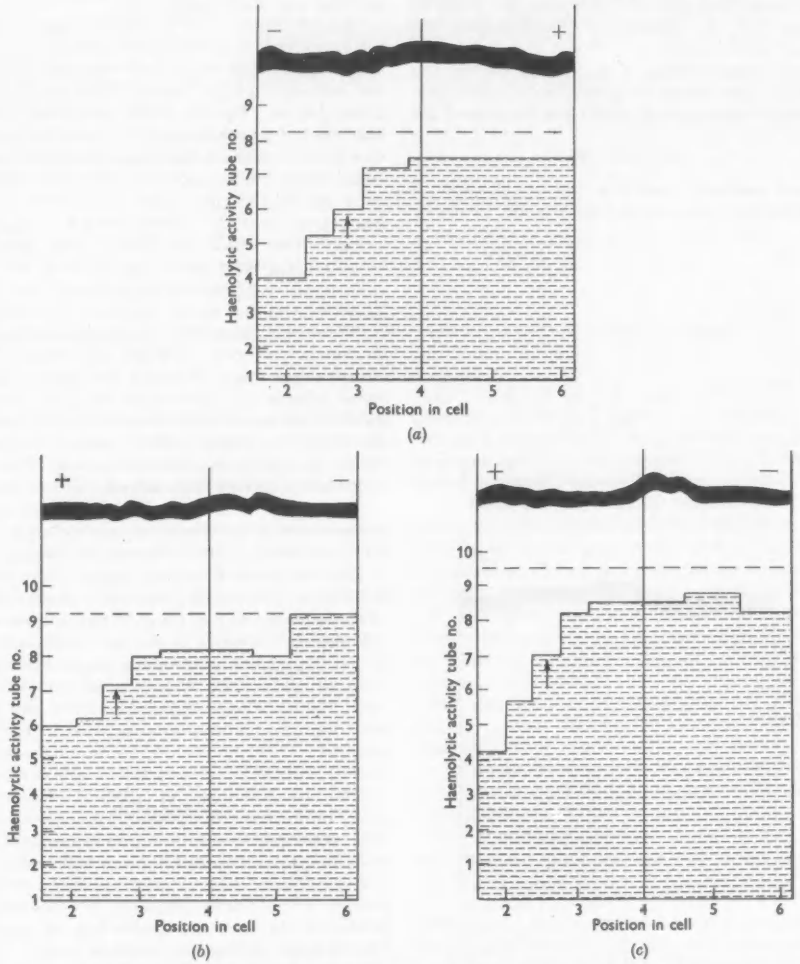

Fig. 2. Diagram of the schlieren patterns and the haemolytic activities of the fractions obtained by Tiselius electrophoresis. The continuous vertical line indicates the position of the original boundary; the broken horizontal line indicates the haemolytic activity of the original solution before electrophoresis; the arrow indicates the fraction having 25% activity. The left-hand side of the original boundary was the pure buffer side. Phosphate buffers, I 0·075. (*a*) pH 6·0, descending limb, 213 min.; (*b*) pH 6·9, ascending limb, 181 min.; (*c*) pH 8·0, ascending limb, 120 min.

obtained, i.e. the active fraction was at a pH above its isoelectric point.

It must be admitted that the mobility figures obtained may not be entirely accurate for the haemolytic factor, although this should, of course, not affect the value for the isoelectric point. The mobility figure refers to a sample of 0·12 cm.³ and this volume occupies a height of 4 mm. in the limb of the electrophoretic cell. This sets the limits of accuracy for the estimation. Furthermore, the 25 % haemolysis arbitrarily chosen may not be the optimum indicator effect. However, had a lower concentration been chosen the possibility of adsorption on to some other protein might have increased the error.

DISCUSSION

Chemical methods used for the purification of staphylococcal α-haemolysin have failed, probably because other materials present have properties closely similar to those of the toxin. The use of less complex media for the growth of the organism should make the fractionation methods more satisfactory. For this reason the medium described by Bramann & Norlin (1951) (a yeast diffusate–casein hydrolysate mixture) was tried, but only very low yields of toxin were obtained, and the medium was abandoned.

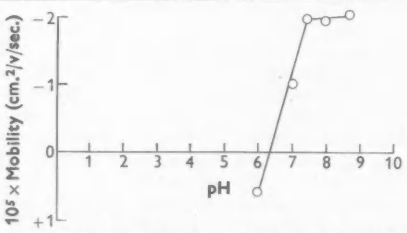

Fig. 3. Isoelectric point of haemolytic factor by Tiselius electrophoresis in phosphate buffers, *I* 0·075.

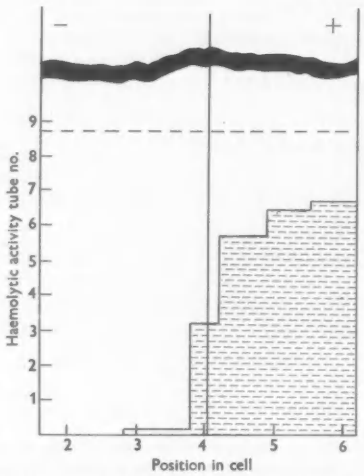

Fig. 4. Diagram of the schlieren pattern and the haemolytic activities of the fractions obtained by Tiselius electrophoresis in phosphate buffer, *I* 0·075, pH 6·95, descending limb, 261 min.

The differential effect of the various reagents on the haemolytic and lethal activities of the preparations suggest that the two activities are not due to the same groupings. Such differences have been noted before. Burnet (1929), although believing that the α-haemolysin and the lethal activity are due to one and the same substance, stated that the lethal effect was completely lost on the formation of a toxoid, whereas the filtrate still retained haemolytic activity. Burky (1933*a*, *b*) reported a sequence in which the lethal and haemolytic activities appeared in culture filtrates, the lethal effect appearing first, the haemolytic effect later or, in some cases, not at all. Avery, Rigdon & Johlin (1937) showed that lithium chloride had no effect on haemolysis but inhibited the dermonecrotic effect of the toxin. Whether the haemolysin and lethal effects are associated with the same or different molecules is still an open question, but the electrophoretic fractionation method may in the future be able to provide the answer. For this, a preparation of very high activity will be required, which so far is not available. Meanwhile, the electrophoretic-fractionation method does allow a limited study of the properties of the toxin.

The results obtained by paper electrophoresis indicate how misleading this method may be. The toxin appeared to run towards the cathode at any pH tested, but never as far as the dextran, thus giving the impression of being negatively charged, even at pH 5. In fact, the haemolysin must have been strongly adsorbed on to the paper, with stronger adsorption at the higher value of pH.

SUMMARY

1. Attempts to purify staphylococcal α-haemolysin by means of ammonium sulphate, ethanol and Cellosolve failed to give preparations of very high purity or activity. Ammonium sulphate was relatively harmless but limited in its powers of fractionation. Cellosolve, in contrast with ethanol, effected a further step in purification.

2. Comparison of the ratios of the lethal to haemolytic activities of the preparations indicate a differential denaturation of the active factors. Support is given for the view that there is a multiplicity of active factors.

3. The isoelectric point of the haemolytic factor was determined by an electrophoretic-fractionation method and found to be at pH 6·4.

I would like to thank Professor S. D. Elek for his interest and for many valuable discussions. I am also grateful to Mr A. Coddington for certain of the nitrogen determinations, and to Miss J. Holmes, Miss S. Williams and Miss J. Goldston for technical assistance during the course of this work.

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Studies on Gastric Proteolysis

1. THE PROTEOLYTIC ACTIVITY OF HUMAN GASTRIC JUICE AND PIG AND CALF GASTRIC MUCOSAL EXTRACTS BELOW pH 5

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Sørensen (1909), Michaelis (1914) and Northrop (1922) found that purified pepsin preparations digest proteins *in vitro* with a single maximum in the region of pH 2. Because of this, most contemporary physiological texts state that gastric proteolysis *in vivo* in mammals occurs most readily near this pH. It was observed as early as 1909, however, that gastric juice and mucosal extracts exhibited relatively greater proteolytic activity at pH 4 than did preparations of pepsin (Takemura, 1909; Hirayama, 1910), and Willstätter & Bamann (1929) reported the existence in swine gastric mucosal extracts of a second proteolytic enzyme, a cathepsin, which was not present in gastric juice and which digested gelatin and egg albumin with a pH maximum at 3·5–4·0. A proteolytic peak at this pH was also demonstrated with both Cudahy and Northrop pepsin, when acting on casein, gelatin and edestin (Dyckerhof & Tewes, 1933), and it was again suggested that a second enzyme was responsible. Norris & Elam (1940) found that crystalline salmon pepsin, when prepared by different methods, gave pH–activity curves with either one or two maxima. These observations strongly suggested that gastric mucosal extracts might exhibit two proteolytic pH maxima, one of which may sometimes disappear during the purification of pepsin.

Not until 1940 was it first demonstrated that human gastric juice may digest proteins with two pH maxima, near 2 and 4 (Freudenberg, 1940). Because activity at the second maximum could be increased by incubating with hydrogen sulphide or hydrogen cyanide, the presence of a cathepsin was again postulated. Buchs (1947) extended Freudenberg's observations, working mainly with human gastric juice on edestin, and concluded that gastric juice contained two enzymes, pepsin and cathepsin. The latter was found, unlike pepsin, to be activated by potassium cyanide, hydrogen sulphide and Co^{2+} and Mn^{2+} ions, to be relatively resistant to ultraviolet radiation and to heating at 70°, and to be inhibited by uranyl and Al^{3+} ions. Two pH maxima, near 2 and 4, have also been observed for proteolysis by human gastric juice by Merten & Ratzer (1949), Milhaud & Epiney (1951), and Bramstedt & Krüger (1954), for proteolysis by swine pepsin (Pope & Stevens, 1951; Ramer, 1954) and for proteolysis by dog gastric juice (Penitschka, 1953; Pfisterer, 1955).

In the calf also, gastric mucosal extracts have been shown to digest proteins with two pH maxima near 2 and 3·5 (Berridge, 1945). The second maximum is attributed, however, to rennin, which when crystallized from crude extracts undoubtedly digests proteins with a pH maximum near 3·5 (Berridge, 1945; De Baun, Connors & Sullivan, 1953). Rennin might thus seem to be taking the place of the 'cathepsin' of other mammals, but the absence of a second pH maximum from rennin-free extracts of calf gastric mucosa has never been demonstrated.

The existence of two pH maxima and of a separate gastric cathepsin have been challenged by several workers. Geilenkirchen & Elbers (1950), using hydrochloric acid extracts of swine mucosa, found two pH maxima with edestin as substrate, but only one with each of four purified plasma-protein fractions. They concluded that the two maxima were the property of the substrate, not of the enzyme. Crebolder, Engel & Otten (1951), working with human gastric juice and the substrates edestin, haemoglobin and plasma protein, contested several of Buchs's observations about the different effects of activators and inhibitors on cathepsin and pepsin. Milhaud & Epiney (1951), although finding two pH maxima, obtained no activation at pH 4 by –SH groups or $-CN^-$ ions. Tolckmitt (1954) concluded that the occurrence of two peaks in the pH–activity curves of Buchs was an artifact caused by changing from hydrochloric acid to acetic acid for control of pH. More recently, Masch & Huchting (1955) and Christensen (1955) have failed to find evidence of more than one pH maximum. Acceptance of the existence of a separate cathepsin in gastric juice or mucous membrane is made still more difficult by the failure of several workers (Buchs, 1947, 1954; Milhaud & Lepine, 1949; Merten, Schramm, Grassmann & Hannig, 1952; Heinrich, 1953) to isolate it separately in solution from gastric mucosa by means of chemical, electrophoretic or ultracentrifugal methods.

There would thus appear to be doubt whether gastric juice and pepsin preparations display one or two proteolytic pH maxima *in vitro*; whether, when two occur, one of them is an artifact associated with the use of certain substrates and buffers; and whether the second maximum, if genuine, is caused by a second enzyme, a cathepsin. The relationship of the proteolytic activity of rennin to the alleged catheptic activity also needs clarifying. An investigation bearing on some of these problems is now described.

EXPERIMENTAL

Gastric juice. Normal human gastric juice was obtained by intragastric tube from healthy human subjects or from hospital patients in whom there was no evidence of anaemia, gastrointestinal disease or disease of the gall-bladder. Subcutaneous histamine or intravenous insulin were the usual stimulants. During collection of gastric juice, saliva was expectorated into a separate vessel. Samples of gastric juice containing bile were discarded.

Gastric mucosal extracts. Hog mucosa was obtained from stomachs removed within 15 min. of death. Extracts were made in 2% (w/v) NaCl unless otherwise stated. For experiments on calf-mucosal extracts, commercial rennet—a glycerol–saline extract of calf mucosa—was used. Extracts were also made from dried hog stomach by using two commercial sources, Pepsac (Boots Pure Drug Co., Ltd.) and Gaster siccata B.P.C. (British Drug Houses Ltd.).

Pepsin. Crystalline swine pepsin (Armour) was used. Pepsin A was prepared from it by the method of Herriott, Desreux & Northrop (1940) as modified by Baker (1951).

Substrates. All but two were obtained commercially. In each instance the purest available product was sought and used, unchanged. The number of components in protein substrates was ascertained by paper electrophoresis at pH 8·6 in 0·1 M-diethyl barbiturate buffer and at pH 7·4 in 0·067 M-phosphate buffer. Human plasma was obtained in batches of about 500 ml. after separation of red cells from blood donated to the Transfusion Service. Benzyloxy-carbonyl-L-glutamyl-L-tyrosine was kindly prepared by Dr P. W. Kent. Plasma was used as substrate in the earlier experiments because, as it consists of several different proteins, there seemed more likelihood of detecting with it all the proteolytic activity of gastric juice than if a single protein substrate were used.

Buffer solutions. Enzyme–substrate mixtures were brought to a suitable pH for digestion by addition of one of the buffers, 0·1 M-glycine–HCl for pH 1–4, or 0·2 M-sodium acetate–HCl for pH 1–5·5. These buffers caused no detectable activation or inhibition of proteolysis when compared with digestion at similar pH in dilute HCl. It was found that at all practicable concentrations of buffer and substrate protein, the pH of the digestion mixture was different from that of the buffer alone, i.e. the substrate becomes a part of the buffer system.

Determination of pH. The pH of the digestion mixtures was determined by the Cambridge or Marconi pH meters with a calomel half-cell and glass electrode. A standard pH of 3·97 at 15° was given by an aqueous 0·05 M-solution of potassium hydrogen phthalate (10·211 g./l.). All proteolytic experiments were carried out at 37°, but the pH of digestion mixtures was determined at room temperature without correction, for the error thus introduced was small (Clark, 1928) compared with the change of pH during proteolysis.

Change of pH *during proteolysis.* This depends on the time of digestion and on the amount of buffer solution used, but with excessive buffer the subsequent formol titrations become large and unmanageable. In practice it was found best to add sufficient buffer to limit the change of pH to less than 0·1 unit.

Determination of amount of proteolysis. The liberation of carboxyl groups from peptide bonds was determined by formol titration. Maximal accuracy was achieved by titrating, without preliminary neutralization before adding formaldehyde, to an end point of pH 8·5 determined by phenolphthalein (Taylor, 1956, 1957). The final formaldehyde concentration was 6–9% (w/v) (Levy, 1934).

Detailed experimental procedure. Boiled gastric juice was found impracticable for control experiments because its cloudiness prevented accurate comparison with the digest at the end point and because on boiling there was a demonstrable loss of $-NH_2$ groups. Unboiled gastric juice has itself a perceptible formaldehyde titration, which increases when gastric juice is incubated alone at acid pH (Taylor, 1956). Thus the use of a digestion mixture at zero time as a control is also unacceptable. An attempt to overcome these difficulties was made by incubating gastric juice and substrate separately in buffer for the same length of time as the digest and using their combined formol

titration as control. This combined value was, however, consistently higher than that of the 'digest' when there was no digestion, and investigation showed the cause to be a small but definite loss of titratable groups on mixing gastric juice and protein substrates before incubation (Taylor, 1956).

The following experimental procedure was finally found acceptable—for each point on the pH–activity curve, seven boiling tubes were prepared and incubated at 37°.

Tube	Substrate (ml.)	Enzyme (ml.)	Buffer (ml.)
1, 6	*a*	—	*x*
2, 7	—	*b*	*y*
3, 4, 5	*a*	*b*	$x+y$

The amounts *x* and *y* of buffer are such as to bring the gastric juice and substrate to the same pH, within 0·1 pH unit. Tube 5 is removed half-way through incubation for the determination of pH. Four other tubes are prepared for each point on the pH–activity curve, each containing sufficient formaldehyde, adjusted to pH 8·5 with 0·1 N-NaOH, to give a formaldehyde concentration in the titration mixture at the end point of 6–9% (w/v). At the end of the digestion period, tubes 1 and 2 are removed together, tube 2 is emptied into tube 1 and approximately one-third of the contents of a formaldehyde tube added rapidly. The remaining two-thirds of the formaldehyde is used to rinse out tube 2 twice into tube 1. In this way a control tube is prepared, each of the constituents of which has been treated exactly as in the digest, even to the initial mixing before the reaction commences. The contents of the control tube are then titrated rapidly with 0·1, 0·04, or 0·0286 N-NaOH until pH 8·5 is obtained. The tube is then stoppered. Neutralized formaldehyde and phenolphthalein are next added to tube 3 and the contents titrated to the colour of tube 1. Tube 4 is treated like tube 3 and tubes 6 and 7 like tubes 1 and 2. Each point on the pH–activity curve is thus determined in duplicate. A little formaldehyde solution is left behind in tubes 2 and 7, but this does not introduce an error since the formaldehyde was initially brought to pH 8·5, i.e. to the final pH of the titration. Duplicates usually agree to within 0·02 ml. and any experiment in which they differ by more than 0·04 ml. is discounted. Known amounts of different amino acids and peptides have been determined under conditions simulating proteolysis (Taylor, 1957) and the standard deviation has never exceeded 0·01 ml. of NaOH.

Criteria for establishing the existence and significance of a pH maximum. In the tables and figures which follow, the number, position and magnitude of the pH maxima have been ascertained from pH–activity curves, which have been constructed by drawing smoothly flowing curves through the experimental data. 'Shoulders' have rarely been encountered in these curves, and when they have occurred they have not been counted as maxima. The significance of a maximum when determined in this way may be assessed as follows: The maximum standard deviation of a point on a pH–activity curve, based on a single observation, is 0·01 ml. of 0·1 N-, 0·04 N- or 0·0286 N-NaOH. The standard error of the difference of two means of two observations each may therefore be calculated and is 0·01 ml. It was thus considered significant if the maximum digestion differed from the minima on either side by more than four times this amount, and this criterion has been adopted throughout.

Reproducibility of pH maxima. When several proteolytic pH–activity curves are obtained by incubating the same gastric juice or mucosal extract with the same substrate under identical conditions on different occasions, the positions of the resulting pH maxima do not vary by more than 0·2 pH unit and often by less. This is illustrated by the first three observations of Table 3.

Extent of substrate hydrolysis. When determining a pH–activity curve it is essential that excess of substrate over enzyme be present, lest maximal hydrolysis should occur over a wide pH range, resulting in a plateau rather than in one or more maxima. Care has been taken to see that the liberated amino acid nitrogen did not usually exceed 15% of that of the substrate, so that even if pepsin can only release about one-third of the peptide nitrogen in proteins (Northrop, Kunitz & Herriott, 1948) an ample excess of substrate has always been present.

RESULTS

Proteolytic activity of human gastric juice

Action on plasma. Digestion of the proteins and peptides of plasma at 37° for periods of ½–6 hr. has been followed over the pH range 1·3–5·0 on twenty-four occasions in seventeen normal subjects, all of whom secreted gastric juice containing free acid. Representative curves relating pH to proteolytic activity are shown in Figs. 1 and 2, and Table 1 indicates the observed pH maxima and the activity in terms of liberated amino acid nitrogen at these maxima for digestion periods of 3 hr. No maxima occurred between pH 4 and 5.

It will be seen that, in all but three subjects, two separate peaks of activity were observed, one at pH 1·7–2·4 and another at pH 3·3–4·0. Of the three exceptions, two subjects showed a third peak at pH 2·7 and 2·8, and the third subject showed a similar peak at pH 2·8 but no peak between pH 3·3 and 4·0. Of the two main maxima, that at pH 1·7–2·4 was the higher in ten subjects but the lower in four. The ratio of maximal activity at the pH 1·7–2·4 peak to that at pH 3·3–4·0 varied from 0·79 to 2·4, mean 1·21. The variation in pH of the maxima from subject to subject is noteworthy. Differences between the batches of plasma used as substrate may partly account for this, but is unlikely to be the main factor because, in subjects 1 and 3 for example, the differing pH maxima were obtained with the same sample of plasma, and because a similar variation of pH maximum occurred with other protein substrates (Table 2) of which the same sample was used throughout. Samples of gastric juice collected at different times from the same individual gave pH maxima which sometimes differed more widely from each other (e.g. subjects 2 and 4) than would be expected from the known range of reproducibility of the pH maxima when using a single sample of gastric juice. No consistent differences of pH maximum could be detected

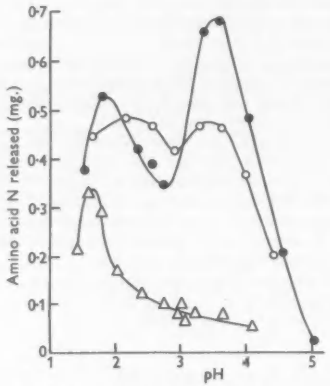

Fig. 1. pH–activity curves for the digestion of proteins by human gastric juice at 37° for 3 hr. ○, Human plasma protein; ●, human serum albumin; △, egg albumin.

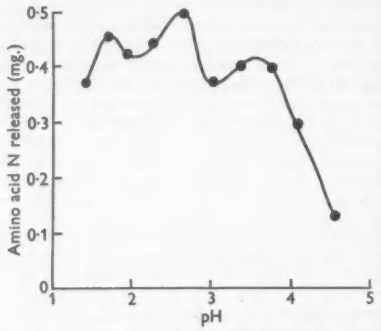

Fig. 2. pH–activity curve, showing 3 maxima, for the digestion of human plasma protein by normal human gastric juice at 37° for 3 hr.

Table 1. pH *maxima for the digestion of plasma protein by gastric juice from seventeen normal subjects*

In this and succeeding tables, the amounts of liberated amino acid nitrogen are those in the whole digest, which usually consisted of 3 ml. of buffer, 1 ml. of enzyme extract or gastric juice, and 1 ml. of plasma or other substrate. With casein and gelatin 2 ml. of a 2% solution was normally taken. Thus with 5% serum or egg albumin as substrate the initial substrate concentration in the digest was normally 1%.

		pH 1·5–2·4		pH 2·5–3·2		pH 3·3–4·0	
Subject	Gastric juice	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
1	Resting juice	1·9	0·60	—	—	3·5	0·25
	Insulin juice	2·1	0·87	—	—	3·8	0·68
2	Resting juice	2·1	0·72	—	—	4·0	0·67
	Resting juice	2·0	0·94	—	—	3·7	1·06
	Insulin juice	2·2	1·00	—	—	3·7	0·85
	Insulin juice	2·2	0·92	—	—	3·5	0·92
3	Insulin juice	1·7	1·01	—	—	3·7	1·11
4	Resting juice	1·8	0·83	—	—	3·5	0·77
	Insulin juice	2·3	0·74	—	—	3·8	0·55
	Insulin juice	2·2	0·41	—	—	3·8	0·36
	Insulin juice	2·4	0·43	—	—	3·6	0·29
5	Histamine juice	2·2	0·73	—	—	3·5	0·63
6	Histamine juice	1·9	0·62	2·8	0·60	Declining activity no maximum	
7	Histamine juice	2·3	0·48	—	—	3·4	0·48
8	Resting juice	1·8	0·57	2·8	0·53	3·8	0·49
9	Histamine juice	2·3	0·73	—	—	3·6	0·83
10	Histamine juice	2·0	0·73	—	—	3·5	0·63
11	Histamine juice	1·8	0·49	—	—	3·5	0·43
12	Histamine juice	1·7	0·45	2·7	0·49	3·6	0·41
13	Histamine juice	1·8	0·53	—	—	3·6	0·67
14	Histamine juice	2·4	0·77	—	—	3·3	0·84
15	Histamine juice	1·9	0·63	—	—	3·4	0·60
16	Histamine juice	1·9	0·59	—	—	3·3	0·57
17	Histamine juice	1·8	0·50	—	—	3·4	0·44

between gastric juice collected after resting, and after administration of histamine or of insulin. The duration of digestion from ½ to 6 hr. did not significantly affect the position and relative magnitude of the pH maxima. The positions of the maxima were unaffected also within experimental error by changes of substrate concentration up to eightfold.

Action on casein, gelatin and edestin. The digestion of casein below pH 5 was followed in three subjects, all of whom showed two maxima, at pH 1·8–2·2 and pH 3·5–3·6 (Table 2). The variation from subject to subject of the pH of the former maximum should be noted although the same sample of casein was used throughout. This sample contained two main components on paper electrophoresis. Gelatin and edestin similarly contained two main electrophoretic components and were each digested with two pH maxima (Table 2). The liberation of titratable groups from gelatin was small compared with that from serum albumin or plasma, in agreement with the results of Northrop (1932).

Action on human and bovine serum albumin. These substrates contained only one protein component when examined by paper electrophoresis, but were each digested with two pH maxima, which occurred within the same range as those for plasma (Fig. 1, Table 2).

Action on egg albumin. This substrate was found on paper electrophoresis to contain two major and two minor components. Its digestion by gastric juice was observed in seven subjects with results somewhat different from those so far obtained (Table 2). Only one maximum of proteolysis, at pH 1·5–1·8, occurred in five subjects (Fig. 1). The maximum at pH 3·3–4·0, obtained with other substrates, did not occur. In the other two, two maxima were observed, one at pH 1·5 and 1·6 and the other at pH 2·5 and 2·8. These were the two subjects who had shown three maxima for the digestion of plasma protein.

Action on benzyloxycarbonyl-L-glutamyl-L-tyrosine. This compound is one of the few synthetic

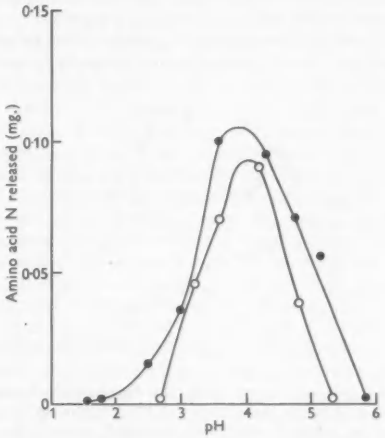

Fig. 3. pH–activity curves for the digestion of benzyloxycarbonyl-L-glutamyl-L-tyrosine by normal human gastric juice (●) and by an extract of normal human fundal mucosa (○) at 37° for 21 hr.

Table 2. pH *maxima for the digestion of protein substrates by gastric juice from normal subjects*

		pH 1·5–2·4		pH 2·5–3·2		pH 3·3–4·0	
Substrate	Gastric juice	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
2% casein	Histamine juice	2·0	0·31	—	—	3·5	0·36
	Histamine juice	1·8	0·21	—	—	3·5	0·11
	Insulin juice	2·2	0·32	—	—	3·6	0·29
5% human serum albumin	Histamine juice	1·9	0·53	—	—	3·5	0·67
	Histamine juice	2·0	0·27	—	—	3·5	0·31
	Histamine juice	2·2	0·62	—	—	3·4	0·50
	Resting juice	2·3	0·24	—	—	3·7	0·27
5% bovine plasma albumin	Histamine juice	2·3	0·67	—	—	3·3	0·63
	Histamine juice	2·0	0·60	—	—	3·3	0·66
2% gelatin	Histamine juice	2·1	0·08	—	—	3·3	0·06
	Histamine juice	2·4	0·11	—	—	3·9	0·07
2% edestin	Histamine juice	1·6	0·14	—	—	4·0	0·10
5% egg albumin	Resting juice	1·8	0·27				
	Histamine juice	1·6	0·35				
	Histamine juice	1·7	0·34	Slowly declining activity, no peak			
	Histamine juice	1·5	0·42				
	Histamine juice	1·7	0·38				
	Histamine juice	1·6	0·35	2·5	0·34	—	—
	Histamine juice	1·5	0·35	2·8	0·20	—	—

dipeptides which pepsin will attack (Fruton & Bergmann, 1939; Harington & Pitt-Rivers, 1944). Significant splitting takes place only after several hours and these authors used incubation periods of as long as 24 hr. in order to demonstrate it. They found that pepsin preparations split this substrate at an optimum pH of 4·0. The effect of gastric juice has not previously been investigated. Fig. 3 shows that the pH optimum for the action of gastric juice on this substrate is at pH 4·0 also. The small amount of hydrolysis over a period of 21 hr. resembles the activity found with pepsin.

Action on other substrates. Gastric juice failed to hydrolyse, at pH values below 6, clupeine, peptone, glycylglycine, L-leucylglycine, L-tyrosylglycine, L-alanylglycine, glycylglycylglycine, L-leucylglycylglycine, L-alanylglycylglycine, glutathione and pteroyl tetraglutamate.

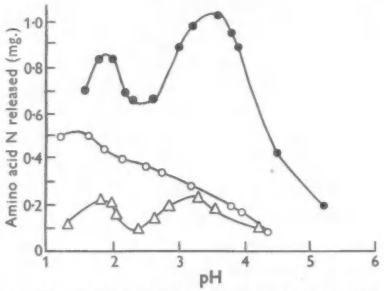

Fig. 4. pH–activity curves for the digestion of proteins by pig gastric mucosal extracts at 37° for 3 hr. ●, Human serum albumin and fresh mucosal extract; ○, egg albumin and dried mucosal extract; △, casein and dried mucosal extract.

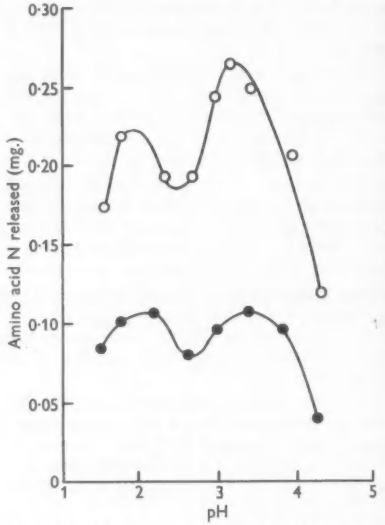

Fig. 5. pH–activity curves for the digestion of human plasma protein by commercial rennet (○) and by the supernatant material after rennin has been removed from rennet (●) at 37° for 3 hr.

Table 3. pH *maxima for the digestion of protein substrates by extracts of swine and calf gastric mucosa*

		pH 1·5–2·4		pH 2·5–3·2		pH 3·3–4·0	
Substrate	Extract	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N	Maximum pH	mg. of amino acid N
Plasma	Pepsac in 0·01 N-HCl	—	—	2·8	1·08	3·6	1·04
		—	—	2·8	1·12	3·6	1·05
		—	—	2·8	0·99	3·6	0·92
	Gaster siccata in 0·01 N-HCl	—	—	2·7	0·46	3·6	0·35
	Commercial rennet	1·9	0·22	—	—	3·3	0·27
	Commercial rennet	1·6	0·42	—	—	3·3	0·36
5% human serum albumin	Fresh swine mucosa in 2% NaCl extract 1	1·8	0·76	—	—	3·6	1·32
	Fresh swine mucosa in 2% NaCl extract 2	1·9	0·84	—	—	3·5	1·04
	Fresh swine mucosa in 2% NaCl extract 3	1·9	0·61	—	—	3·8	0·85
	Pepsac in 2% NaCl	1·9	0·20	—	—	3·3	0·22
		2·0	0·73	—	—	3·3	0·90
2% casein	Pepsac in 0·01 N-HCl	2·1	0·15	—	—	3·3	0·18
	extract	1·9	0·21	—	—	3·3	0·23
	Gaster siccata in 0·01 N-HCl	—	—	2·6	0·25	3·7	0·38
5% egg albumin	Gaster siccata in 2% NaCl	1·5	0·51	Declining activity without maxima			
	Gaster siccata in 0·01 N-HCl	1·7	0·20	Declining activity without maxima			
	Commercial rennet	1·6	0·14	—	—	3·7	0·03

Proteolytic activity of swine and calf mucosal extracts

Extracts of fresh pig mucosa and of dried whole stomach, in sodium chloride solution or in 0·01 N-hydrochloric acid, digested plasma, casein and human serum albumin with two pH maxima, at pH values ranging from 1·8 to 2·8 and from 3·5 to 3·8 (Fig. 4) (Table 3). Egg albumin was digested with only a single maximum at pH 1·5. These results resemble those with human gastric juice. Two samples of commercial rennet also digested plasma with two pH maxima at pH 1·6 and 3·3, and 1·9 and 3·3 respectively (Fig. 5). Unlike human gastric juice and pig mucosal extracts, rennet digested egg albumin with two pH maxima, at pH 1·6 and 3·7 (Table 3). Digestion at this last maximum was slight. This maximum is at a different pH from the second maximum with this substrate which was observed at pH 2·5 and 2·8 in a minority of normal human subjects.

Effect of substrate and substrate components on pH–activity curves

The optimum near pH 2 for the digestion of proteins by the same sample of gastric juice or pepsin is known to vary slightly with the substrate (Northrop *et al.* 1948) and the present work confirms this (compare maxima with egg albumin and edestin with those with other substrates). That the nature of the substrate may also affect the shape of the pH–activity curve between pH 3 and 4 is already apparent, for, whereas most proteins are digested with a second maximum in this region, egg albumin is not. It is possible that pH–activity curves with two maxima might arise if the substrate were to contain more than one protein component, each being digested at a single but different optimum pH. Although no protein or protein component has yet been found which, when digested by gastric juice, shows a solitary optimum at pH values high enough to account in this way for the observed second maximum at pH 3·3–4·0, the possibility that different substrate components may be responsible for the two maxima must be more positively excluded.

The number of protein components in each of the substrates was determined by paper electrophoresis. Whereas plasma, edestin, casein, gelatin and egg albumin consisted of more than one component, human serum albumin, crystalline bovine plasma albumin and bovine plasma albumin fraction V (Cohn & Hughes, 1946) each appeared to be homogeneous. It is apparent therefore that substrates with only one component give digestion curves with two pH maxima; that plasma, with at least four components, gives similarly two maxima; but that egg albumin with two major electrophoretic components has a single digestion maximum. It would seem therefore that the number of pH maxima varies independently of the number of substrate components.

Even when a protein substrate is electrophoretically homogeneous it is still possible, if some of the molecules become denatured before or during digestion, for two components to be available, the native protein and the denatured, for hydrolysis by gastric juice or mucosal extracts. If each component were to undergo optimum proteolysis at a different pH, the resulting pH–activity curve for the apparently homogeneous substrate might show two maxima. This possibility was investigated by denaturing a 4% solution of plasma albumin fraction V, and a 4% solution of crystalline bovine plasma albumin by boiling for 10 min. at pH 3·0. The resulting solutions contained only one electrophoretic component on paper and were used as substrates for gastric juice and for pepsin A. Both were still digested with two pH maxima below pH 5·0 (Fig. 6). Further evidence that single-component substrates may be digested with two pH maxima is furnished by Fig. 7, which shows the auto-digestion curve of pepsin A. This enzyme preparation has only one protein component on paper electrophoresis and by solubility measurements (Fig. 8), yet digests itself with two pH maxima at 1·8 and 3·3. It would seem therefore that, although the precise maximal points vary with different

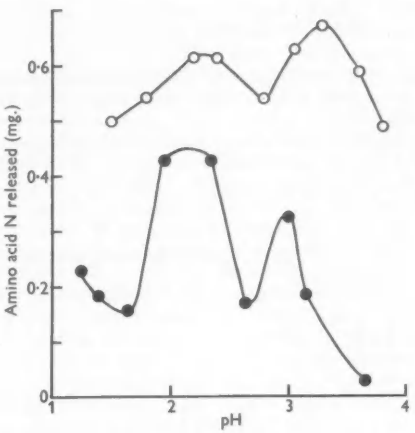

Fig. 6. pH–activity curves for the digestion of heat-denatured bovine plasma albumin fraction V by normal human gastric juice (○) and pepsin A (●) at 37° for 3 hr.

substrates, the number of proteolytic maxima is not related to the number of substrate components or to the presence of denatured substrate.

Effect of buffer anions on pH–*activity curves*

Tolckmitt (1954) claimed that the existence of two pH maxima in the pH–activity curves for the hydrolysis of proteins by gastric juice is an artifact caused in the experiments of several investigators by substitution of acetic acid for hydrochloric acid for control of pH above 2·5. Springer (1950) and Masch & Huchting (1955) have also demonstrated that different anions slightly alter the shape of pH–activity curves.

The principal buffer that has been used in the present experiments is glycine–hydrochloric acid. Fig. 9 shows that when a fresh pig mucosal extract digests plasma albumin V in the presence of hydrochloric acid only, a curve with two maxima is obtained. An almost identical curve is obtained when the same materials are incubated in glycine–hydrochloric acid buffer. The use of the latter buffer is not therefore the cause of the two maxima. Tolckmitt himself, although finding only a single pH-optimum, observed no important differences between digestion curves obtained with hydrochloric acid alone and with glycine–hydrochloric acid. The only other buffer occasionally used in the experiments described here was sodium acetate–hydrochloric acid for pH values above 3·8. Despite the use of this buffer at higher pH values it was still possible to obtain curves with single pH maxima when egg albumin was used as substrate (Figs. 1, 3). Further, whenever double pH maxima were found, they never occurred at pH values related to those over which the second buffer was in use.

Role of rennin

In Fig. 5 are shown the pH–activity curves of crude rennet and of the material remaining after precipitation from it of rennin. Rennin was removed by saturating three times with sodium chloride. After the final saturation a fine opalescence was removed by centrifuging at 38 000 *g* for 15 min. in the Spinco ultracentrifuge. The clear supernatant was free of rennin activity but was nevertheless able to digest plasma protein with

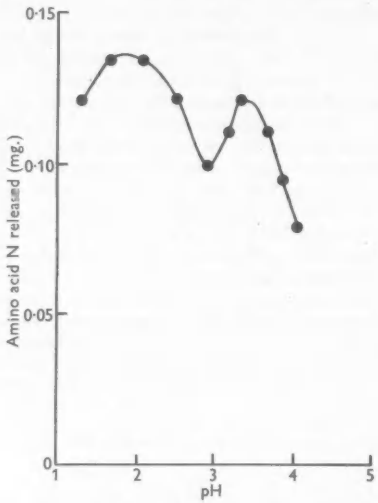

Fig. 7. pH–activity curve for the auto-digestion of pepsin A, prepared from crystalline swine pepsin, at 37° for 3 hr.

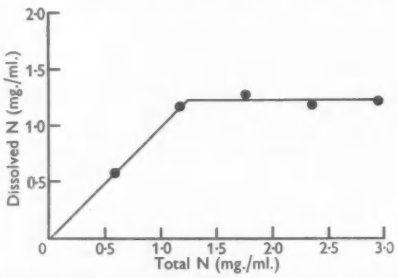

Fig. 8. Solubility curve of pepsin A in 0·2 M-acetate buffer at pH 4·8 and 60 % saturation with magnesium sulphate, at room temperature. Nitrogen was estimated by micro-Kjeldahl technique.

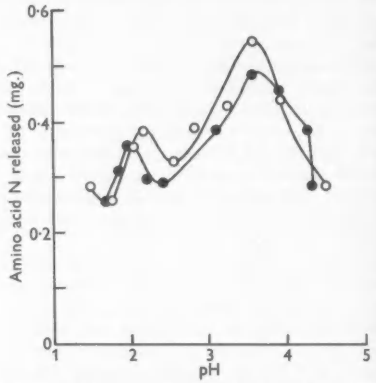

Fig. 9. pH–activity curves for the digestion of bovine plasma albumin fraction V, by a fresh extract of pig fundal mucosa in media containing HCl alone (○) and glycine–HCl (●) for control of pH, at 37° for 3 hr.

maxima at pH 2·0 and 3·5. The single pH optimum of rennin is experimentally well-founded, but in the absence of the enzyme calf mucosal extracts still exhibit two proteolytic pH maxima, which resemble those of human gastric juice and swine mucosal extracts.

DISCUSSION

The results presented above indicate that, under the defined experimental conditions, by the use of unmodified gastric juice or simple gastric mucosal extracts and a method which estimates total liberated carboxyl groups directly, proteins other than egg albumin are digested with two pH maxima below pH 5. Two observations require special emphasis: first, the occasional occurrence of pH–activity curves with three proteolytic pH maxima, which have not been described previously; secondly, the unique position of egg albumin among the substrates so far investigated in yielding, usually, only a single pH maximum.

It has proved possible to exclude the chemical properties of the buffer as the cause of the two maxima. Tolckmitt's contention (1954) that the double pH maxima of previous workers have resulted from changing buffers may be examined more closely. He observes the pH optimum of gastric juice upon edestin to be about 3·0 and finds that at pH 2·5 in M-acetic acid proteolytic activity is about 20% less than in 0·009M-hydrochloric acid. If a change is made from one to the other at this point, a minimum appears which has the effect of creating a more acid maximum at about pH 2 in addition to the maximum at about pH 3 which would in any case occur. These results should be interpreted with caution, for the observed pH optimum of 3·0 for the digestion of edestin is unusually high, and most observers from the time of Sørensen have observed optimum activity at pH values below this, usually between 1·5 and 2·5. Even more striking is the fact that, on changing from hydrochloric acid for control of pH below pH 2·5 to acetic acid above pH 2·5, a new maximum at about pH 2 appears as an artifact. Thus if Tolckmitt be correct, the characteristic pH maximum of gastric juice and pepsin at about pH 2, which has been recognized and repeatedly confirmed over many years, is artifactual. The conditions under which Tolckmitt's artifact appears are not in any case applicable to the experiments that have been described above. A further strong point against either maximum arising through a change of buffer is the occurrence under exactly comparable experimental conditions of only a single optimum with egg albumin and with benzyloxycarbonyl-L-glutamyl-L-tyrosine.

From the evidence presented here multi-component substrates can also be excluded as the cause of a double pH maximum, but the role of the individual substrate is clearly important, otherwise egg albumin and benzyloxycarbonyl-L-glutamyl-L-tyrosine would not behave differently from the others. Equally, however, would it appear that the enzyme source is important, for Norris & Elam (1940) found when using the same substrate that the number of maxima depends on the method of preparation of crystalline salmon pepsin.

The experiments with rennet indicate that calf mucosal extracts, in the absence of rennin, possess a proteolytic action similar to that of human gastric juice. Rennin would thus appear to be present in the calf as a proteolytic enzyme additional to that (or those) responsible for peptic and 'catheptic' activity. The 'catheptic' activity of human gastric juice and of pig mucosal extracts is not therefore the physiological counterpart of rennin in the calf, as the similarity of their proteolytic pH maxima might at first suggest.

It may be wondered how it has happened that so many observers before 1930, and several after that date, have reported pH–activity curves with only a single pH maximum, usually in the neighbourhood of pH 2. There are seven reasons for this, the first five of which have been mentioned by Buchs (1947, 1954): (1) Many experiments in the literature have been carried out on preparations made from commercial pepsin (in which 'catheptic' activity may possibly have been destroyed) and not upon gastric juice. (2) pH–activity curves have often not been continued to as high as pH 4 and, even when they have, insufficient points are available to reveal the maximum between pH 3·3 and 4·0 (see Sørensen, 1909; Rona & Kleinmann, 1924). (3) Different methods of extraction of pepsin may cause differing types of curve in which the maximum between 3·3 and 4·0 is often masked (see Norris & Elam, 1940). (4) An obvious second maximum is sometimes ignored (see Michaelis & Mendelsohn, 1914). (5) Willstätter & Bamann stated in 1929 that cathepsin was not secreted in the gastric juice, although found in the mucous membrane of the stomach, and since that time cathepsin has been regarded as a more or less unwelcome contaminant of pepsin preparations and has been systematically disregarded. (6) Some of the earlier experiments were carried out with egg albumin as substrate (e.g. Sørensen, 1909) and could not, in view of the observations presented here, be expected to yield pH–activity curves with double maxima. (7) There is evidence from the work of Masch & Huchting (1955) that the method of estimation of proteolysis affects the shape of the pH–activity curve. Although most of their pH–activity curves show single maxima, many of those which have been determined for gastric juice

(rather then pepsin) by the biuret method appear to show two. With the Folin and nephelometric methods only single maxima are shown. It therefore becomes particularly important to determine proteolysis by methods such as formol titration, which estimate hydrolysed peptide bonds quantitatively, rather than to rely on methods that estimate only a particular hydrolytic product or the precipitation of undigested substrate.

Several of these seven factors may have operated together to cause the single pH maxima that have been observed by earlier workers. It is difficult, however, to reconstruct in retrospect exactly what has happened, and some observations of curves with only one maximum, such as those with samples of Northrop pepsin, are not easily accounted for by any combination of these factors. The present investigation provides evidence that more than one pH maximum is normally to be found with most substrates, and that the presence of more than one maximum cannot be adequately explained as an artifactual effect of buffer anions or of differing substrate components.

Finally the influence of these observations on contemporary ideas of *in vivo* gastric proteolysis may be considered. From the tables and figures of the text it is apparent that, *in vitro*, proteolysis at the maximum between pH 3·3 and 4·0 could be approximately as effective as that at pH 1·6–2·4. There is therefore no physiological reason why intragastric proteolysis should not begin at as high as pH 4·5 and proceed swiftly at pH values above 3·5. It would thus be possible for significant gastric digestion of proteins to occur in the absence of 'free acid'. Since it takes an appreciable time for the gastric contents to achieve a pH as low as 2·0, it is possible that when the stomach empties early the lower pH maximum of gastric proteolysis is never reached, but this need not mean that significant gastric proteolysis has not occurred. Similarly, when bile-stained duodenal contents are regurgitated into the stomach, gastric proteolysis will not cease unless the pH rises above 4·5.

SUMMARY

1. The gastric juice of twenty-five normal subjects was found to digest one or more of the substrates plasma protein, casein, human serum albumin, gelatin, bovine plasma albumin and edestin with two pH maxima below 5. The two maxima range from pH 1·6 to 2·4 and from pH 3·3 to 4·0 for different individuals and with different substrates.

2. The pH maxima of gastric juices from different subjects and of samples collected at different times from the same individual sometimes differed more widely, when tested on the same sample of substrate, than could be accounted for by the range of reproducibility of the pH maxima as determined with the same sample of gastric juice.

3. The two optima are unaffected, within experimental error, by time of digestion from ½ to 6 hr. or by changes of substrate concentration up to eightfold.

4. The gastric juice of two normal subjects digested blood plasma with three pH maxima, the third occurring at pH 2·8 and 2·9.

5. In contrast with the action on most substrates, the gastric juice of five normal subjects digested egg albumin with only one maximum at pH 1·5–1·8. The gastric juice of the two subjects who digested plasma with three maxima digested egg albumin with two, the second maximum occurring at pH 2·5 and 2·8.

6. The gastric juice of two normal subjects hydrolysed the specific pepsin substrate, benzyloxycarbonyl-L-glutamyl-L-tyrosine, with one optimum at pH 4·0.

7. Swine and calf gastric mucosal extracts possessed proteolytic properties similar to those of human gastric juice, except that calf extracts digested egg albumin with two maxima.

8. The proteolytic activity of calf mucosal extracts at pH 3·5 is not caused wholly by rennin, which has a pH maximum in this region, but also by another enzyme which is present in extracts from which rennin has been removed.

9. The two proteolytic pH maxima are shown to occur independently of the number of substrate components and of the buffer anions present in the digestion mixture.

10. Gastric proteolysis *in vivo* might occur readily at pH 3·5–4·5. It is not necessary that an intragastric pH as low as 2·0 or 1·5 be achieved.

I wish to thank Miss Barbara Hunt and Mr B. Collett for skilful assistance with part of this work and the many patients and subjects who have given gastric juice, particularly Dr A. Bowyer. I am grateful to Dr P. W. Kent for the preparation of benzyloxycarbonyl-L-glutamyl-L-tyrosine, to Dr R. B. Fisher for statistical advice, to Mr J. R. P. O'Brien for his helpful interest, and to both Dr Fisher and Mr O'Brien for reading and criticizing the D.M. Thesis from which the material of this paper is abridged.

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Studies on the Proteins of Fish Skeletal Muscle

6. AMINO ACID COMPOSITION OF COD FIBRILLAR PROTEINS*

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Recent work (Connell, 1958*b*) has shown that the peculiar instability of cod as compared with rabbit myosin cannot be explained with reference to the picture of the molecular weight or shape of the fish protein revealed by sedimentation, diffusion and viscosity. It was therefore decided to see whether any features of the amino acid composition of cod myosin distinguished it from rabbit myosin.

The other main fibrillar protein, actin, has been included in the investigation since it was also of interest to compare the amino acid composition of this protein from two different species. In addition, cod 'natural' actomyosin, that is actomyosin prepared directly from cod muscle, has been analysed both because of its importance as a model substance and because an idea of the relative amounts of myosin and actin in the complex can be obtained by comparing their amino acid compositions. Since the method of Hasselbach & Schneider (1951) cannot be applied to fish because the solution used to extract myosin also extracts considerable amounts of actin (J. J. Connell, unpublished work), the indirect determination of myosin and actin in natural actomyosin would seem to be the only way of measuring the amounts of myosin and actin in fish muscle.

* Part 5: Connell (1958*b*).

MATERIALS AND METHODS

Proteins. Myosin, actin and actomyosin were prepared from the skeletal muscle of cod (*Gadus callarias* L.) killed just beforehand as described by Connell (1958*b*). Myosin and actomyosin were precipitated from the final buffer solution by dialysis at 0° against many changes of distilled water for several days, and the precipitates were dried with acetone, ethanol–ether (1:3, v/v) and finally in air at room temperature. The actin was purified twice by removing unpolymerized material in the preparative ultracentrifuge, the final pellet of F-actin being depolymerized in 0·1 mM-adenosine triphosphate solution, pH 8 (1 ml./10 mg. of protein), and salt removed by dialysis against distilled

water for 4 days at 0°. The protein was then precipitated by adding 0·1 N-HCl until the pH of the solution was about 4; the precipitate was washed twice with acetone and then with ethanol–ether (1:3, v/v); it was finally dried in air at room temperature.

The preparations of myosin, actin and actomyosin had 0·97, 0·29 and 1·94% of ash and 16·41, 16·40 and 16·50% of nitrogen (moisture- and ash-free), respectively.

Hydrolysis. Samples (30–50 mg.) of the proteins were weighed into 8 mm. × 75 mm. Pyrex test tubes and 0·5 ml. of freshly distilled constant-boiling HCl was added to each. The mixture was then frozen and the tube evacuated with an oil pump and sealed whilst still evacuated. The tubes were heated for 24 or 48 hr. in an oven at 110±0·5°, and the contents filtered to remove humin and made up to volume. The hydrolysates were kept frozen until analysed, when portions were removed and dried over NaOH pellets in a vacuum desiccator before application to the column of ion-exchange resin.

Determination of amino acids. The amino acids were separated by elution chromatography on columns of powdered (finer than 200 mesh) Amberlite IR-120 ion-exchange resin (CG-120, type II as supplied by British Drug Houses Ltd.) according to Moore, Spackman & Stein (1958). Fractions (2 ml.) of eluate were collected and analysed according to Moore & Stein's (1954) method with the exception that stannous chloride (0·08%) was used as the reducing agent.

Tryptophan was determined on samples of the intact protein by the method of Spies & Chambers (1949), and cystine plus cysteine by performic acid oxidation (Schram, Moore & Bigwood, 1954) followed by hydrolysis of the protein and separation of the cysteic acid on a 0·9 cm. × 15 cm. column of IR-120 (Moore *et al.* 1958).

A determination of the amino acids in a 24 hr. hydrolysate of crystalline bovine-serum albumin (Armour Laboratories, Chicago, Ill., U.S.A.) gave a result which was in satisfactory agreement with the published analyses for this protein (Moore & Stein, 1949), the recovery of nitrogen being 100·7%.

RESULTS

The amino acid compositions of cod myosin, actin and 'natural' actomyosin are given in Table 1. The results for threonine, serine and amide nitrogen were obtained by linearly extrapolating to zero time the results for 24 and 48 hr. hydrolyses. The arginine contents of the myosin and actomyosin 48 hr. hydrolysates were about 10% higher than those for the 24 hr. hydrolysates; the former values are given in Table 1. The results for all the other amino acids (except tryptophan and cystine plus cysteine) are the means of determinations on 24 and 48 hr. hydrolysates. Replicates generally agreed within 3%.

Also shown in Table 1 are the amino acid compositions of rabbit myosin and actin, and the percentage of cod myosin in cod actomyosin calculated from the results on each amino acid assuming that myosin and actin are the sole constituents of actomyosin.

DISCUSSION

It is evident from the results shown in Table 1 that the amino acid compositions of cod myosin and cod actin are closely similar to those of the rabbit proteins. Even if the other evidence (Connell, 1954, 1958*a*, *b*) is disregarded there can be little doubt that the cod-muscle proteins whose analyses are presented here are genuine counterparts of the proteins prepared from rabbit muscle. The figures for the cod proteins tend as a whole to be slightly lower than those for the rabbit proteins, particularly for lysine and glutamic acid. Part of this discrepancy probably can be ascribed to the rather low total recoveries of nitrogen of the cod-protein analyses (about 95%). A number of other small differences between the cod and rabbit results may be noted: aspartic acid is lower in cod actin, proline is higher in both cod proteins and amide is higher in cod myosin. A separate determination of the amide nitrogen of cod myosin by the method of Chibnall, Mangan & Rees (1958) gave a value of 81 g.mol. of amide nitrogen/10^5 g. of protein, which is closer to the value for rabbit myosin. The value of the tryptophan content of cod actin is lower than the value for rabbit actin given by Kominz, Hough, Symonds & Laki (1954), but this difference is perhaps not significant since both Tsao & Bailey (1953) and Perry (1947) give the value for rabbit as 6 g.mol. of tryptophan/10^5 g. of protein.

Myosin and actin therefore join the growing number of proteins which when isolated from different sources have amino acid compositions that are very alike; at least for two species, myosin fulfils the prediction which Bailey (1954) made for it in this respect. If differences between the amino acid compositions of cod and rabbit myosins are responsible for the different stabilities of the two proteins then these differences must be small and incapable of being detected by the present methods of amino acid determination.

The values for the proportion of myosin in cod 'natural' actomyosin calculated from each amino acid determination appear at first glance to vary enormously. However, it is clear that, since the error in the determination of each amino acid is approximately the same, the accuracy of the value calculated in this way will depend upon the differences between the quantities of individual amino acids in the myosin and actin. Where these differences are less than 1 g. of amino acid nitrogen/100 g. of protein nitrogen, that is for serine, alanine, valine, methionine, phenylalanine, tryptophan and cystine, the calculated values are so inaccurate as to be useless. If the values calculated from these amino acids are excluded and the remaining values weighted according to the difference between the quantities of individual

Table 1. *Amino acid composition of cod fibrillar proteins compared with rabbit myosin and rabbit actin*

	Cod (g. of N/100 g. of protein N)			Percentage of cod myosin in cod actomyosin $\left(\frac{(3)-(2)}{(1)-(2)} \times 100\right)$	g.mol./10^5 g. of protein					
					Myosin			Actin		
						Rabbit				
	Myosin (1)	Actin (2)	Acto-myosin (3)		Cod	(Kominz, Hough, Symonds & Laki, 1954)	Bailey (1948)	Cod	Rabbit (Kominz, Hough, Symonds & Laki, 1954)	Cod actomyosin
Aspartic acid	7·21	6·08	6·94	76	85	85	67	71	82	82
Threonine	3·04	4·81	4·04	44	36	41	43	56	59	48
Serine	3·87	4·71	4·61	12	45	41	41	55	56	54
Glutamic acid	12·31	7·56	10·94	71	145	155	150	89	101	129
Proline	2·51	4·35	2·60	83	25	22	17	51	44	31
Glycine	3·77	5·51	4·25	73	44	39	25	65	67	50
Alanine	6·03	5·80	6·03	100	71	78	73	68	71	71
Valine	3·75	4·22	3·99	49	44	42	22	49	42	47
Methionine	1·67	2·42	1·20	—	20	22	23	28	30	14
Isoleucine	2·95	4·89	3·42	76	35	42	119	57	57	40
Leucine	6·52	5·50	6·35	83	77	79		64	63	75
Tyrosine	1·26	2·77	1·43	89	15	18	19	32	32	17
Phenylalanine	1·98	2·30	1·96	—	23	27	26	27	29	23
Lysine	12·14	7·37	11·01	76	72	85	81	43	52	65
Histidine	3·33	5·26	3·95	68	13	15	15	20	19	16
Arginine	12·99	14·22	13·00	100	38	41	42	41	38	38
Tryptophan	0·62	1·29	0·75	80	3·7	3·9	3·9	7·6	10	4·4
Cystine/2	0·61	0·97	0·81	45	7·2	8·6	12	11·4	11·2	9·5
Amide N	8·45	5·20	8·15	90	99	86	86	61	66	96
Total	94·65	95·28	95·43							

amino acids in myosin and actin, the values calculated from the lysine and leucine figures receiving the greatest and least weights respectively, the average value obtained is about 73 %. The error in this weighted average cannot be calculated simply but is probably about 6–7 %. The ratio of myosin to actin in cod 'natural' actomyosin is therefore between 2 and 4:1; this is also probably close to the ratio between the two proteins in whole cod muscle since cod 'natural' actomyosin makes up practically the whole of the fibrillar protein of cod (Dyer, French & Snow, 1950) and therefore contains all the myosin and actin of the muscle. It appears that the ratio of myosin to actin in cod muscle is similar to the ratio of close to 3:1 found for rabbit muscle by Hasselbach & Schneider (1951). However, the actual quantities of myosin and actin in cod muscle are likely to be greater than those in rabbit muscle since the amount of fibrillar protein is greater in cod than in rabbit (for summary see Bailey, 1954).

SUMMARY

1. The amino acid compositions of cod myosin, cod actin and cod 'natural' actomyosin have been determined.

2. Cod myosin and cod actin closely resemble the corresponding rabbit proteins in amino acid composition.

3. The ratio of myosin to actin in cod 'natural' actomyosin was found to lie between 2 and 4:1 and therefore approximates to the corresponding ratio in rabbit muscle.

We are indebted to Dr J. Leggat Bailey for communicating in advance of publication Drs Moore, Spackman and Stein's method of amino acid analysis.

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A Quantitative Method for the Chromatographic Separation of 17-Oxo Steroid Sulphates from 17-Oxo Steroid Glucuronides: with Observations on the Behaviour of Conjugated Corticosteroids on the Same System

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Steroid metabolites are excreted in the urine mainly as glucuronide or sulphate conjugates. Although it is possible to distinguish between these two forms of conjugation by specific hydrolytic procedures and to separate them physically by solvent partition after acetylation (Kellie & Wade, 1956), neither process gives the steroid conjugates in unmodified form. The former process liberates free steroids and the latter acetylated glucuronides which can no longer be hydrolysed by β-glucuronidase or oxidized by bismuthate. A method has been evolved in which, by taking advantage of the adsorption of the conjugates on neutral alumina,

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the 17-oxo steroid glucuronides and 17-oxo steroid sulphates are separated quantitatively from each other in unmodified form.

Simultaneous observations have been made of the behaviour of conjugated corticosteroid metabolites, measured as 17-oxogenic steroids (Norymberski, 1952), on the same chromatographic system.

A preliminary report of this work has been published (Barlow, 1957).

METHODS

Extraction of steroid conjugates. Steroid conjugates were extracted from urine by the method of Edwards, Kellie & Wade (1953), in which $(NH_4)_2SO_4$ (50 g./100 ml.) is dissolved in the urine and the conjugates are extracted with ether–ethanol (3:1, v/v) mixture ($3 \times \frac{1}{5}$ of the original urine volume). No adjustment of the urinary pH was necessary for quantitative extraction of 17-oxo steroid conjugates but maximum extraction of 17-oxogenic steroid conjugates (85–92%) was obtained by adjusting the urine to pH 2 before adding $(NH_4)_2SO_4$ (Edwards & Kellie, 1958). The ether–ethanol extracts were combined and filtered before chromatography.

Hydrolysis of steroid conjugates. To discriminate between the various forms of conjugation the following procedures were adopted.

The glucuronides were hydrolysed with ox-liver β-glucuronidase (Ketodase, Warner-Chilcott Laboratories, New York, U.S.A.) at a concentration of 750 Fishman units/ml. of original urine. (One Fishman unit is the amount of enzyme necessary to liberate 1 μg. of phenolphthalein from its glucuronide after incubation of the mixture at pH 4·5 and 38° for 1 hr.) In parallel experiments, limpet (*Patella vulgata*) powder was also used as a source of β-glucuronidase (Dodgson & Spencer, 1953), 0·03 M-KH_2PO_4 being added to inhibit the sulphatases present. The material was dissolved in acetate buffer containing 0·03 M-KH_2PO_4 (pH 5·0 for Ketodase and pH 4·0 for limpet powder) prepared by mixing 0·5 M-sodium acetate with the corresponding volume of 0·5 M-acetic acid. The enzyme and penicillin were added and the hydrolysis was carried out at 37° for 40 hr. According to Wotiz, Lemon, Marcus & Savard (1957) Ketodase contains no sulphatase.

After hydrolysis, liberated 17-oxo steroids were extracted with benzene ($3 \times \frac{1}{5}$ of the hydrolysate volume) and the combined benzene extracts were washed with N-NaOH ($2 \times \frac{1}{5}$ vol.) and with water to neutrality. Portions of this extract were taken for determination by the Zimmermann reaction (Callow, Callow & Emmens, 1938).

Corticosteroid metabolites liberated by enzyme hydrolysis were extracted with $CHCl_3$ ($3 \times \frac{1}{5}$ vol.) and the combined $CHCl_3$ extracts washed with N-NaOH ($1 \times \frac{1}{5}$ vol.) and with water to neutrality. Portions removed for bismuthate oxidation were taken to dryness below 40° and under N_2.

Steroid sulphates in aqueous solution were hydrolysed by adjusting to pH 1 and continuously extracting with ether in a liquid–liquid extractor for 48 hr. (Lieberman & Dobriner, 1948). The ether extract was washed with N-NaOH ($2 \times \frac{1}{5}$ vol.) and with water to neutrality. Portions were removed for determination by the Zimmermann reaction.

When it was unnecessary to discriminate between the modes of conjugation, hot-acid hydrolysis was used to determine the total amount of 17-oxo steroid. The material was dissolved in, or diluted to, 10 ml. with water in a stoppered tube, and conc. HCl (3 ml.) added, and the tube placed in a boiling-water bath for 10 min. After the solution had cooled, the 17-oxo steroids were extracted by shaking with carbon tetrachloride (5 ml.) and this extract was washed with 3N-NaOH (2 ml.) and with water to neutrality (Drekter *et al.* 1952). Portions were taken for determination by the Zimmermann reaction.

Separation of steroid conjugates after acetylation. The known 17-oxo steroid sulphates are not acetylated by pyridine and acetic anhydride, whereas steroid glucuronides contain three free hydroxyl groups on the glucuronic acid residue which are acetylated by this procedure. Acetylation of these groups converts the previously water-soluble glucuronides into water-insoluble acetylated glucuronides which can be separated from the water-soluble steroid sulphates by partition between benzene and water (Kellie & Wade, 1956). This procedure was used to provide further evidence of the mode of conjugation in the various chromatogram fractions.

The dried residue from each fraction was dissolved in pyridine (2 ml.) and acetic anhydride (2 ml.) and allowed to stand overnight at room temperature. The mixture was diluted with ice–water (25 ml.) and the water-insoluble acetylated glucuronides were extracted with benzene (3×10 ml.). The combined benzene extracts were washed with water (2×10 ml.), and dried over anhydrous Na_2SO_4 and evaporated to dryness. Portions were removed and the amount of 17-oxo steroid present as the acetylated glucuronide was determined.

Determination of 17-oxogenic steroid glucuronides. Brooks & Norymberski (1953) introduced a method of estimating corticosteroid metabolites which makes use of the fact that certain 17α-hydroxycorticosteroids can be oxidized by sodium bismuthate to 17-oxo steroids and can be indirectly measured in this way. Such steroids are called 17-oxogenic steroids. This method simultaneously measures 17-oxo steroids present before oxidation; the figure obtained after oxidation is therefore the sum of the 17-oxo steroids present before oxidation and those formed during the oxidation (total 17-oxo steroids). To determine the amount of 17-oxogenic steroid present, an independent determination of the 17-oxo steroids is made and the value obtained by difference.

In the present work the procedure used was a modification of the Brooks & Norymberski method (Edwards & Kellie, 1958), and the values quoted are for 'total 17-oxo steroid glucuronides'.

Material (approx. 20 μg. of 'total 17-oxo steroid') was transferred to a stoppered tube (B19 standard joint, 60 ml. capacity) and dissolved in or diluted with water–ethanol (1:3, v/v; 25 ml.) containing trichloroacetic acid (1 g.). Sodium bismuthate A.R. (2 g.) was added and the mixture agitated in the dark for 2 hr. The reaction was stopped by centrifuging and a portion of the supernatant liquid (20 ml.) pipetted into a separating funnel containing 60 ml. of diluting fluid (3% of Na_2SO_4 anhyd. and 2% Na_2SO_3 anhyd. in water, w/v). The mixture was extracted with dichloromethane (3×10 ml.) and the combined extracts were washed with N-NaOH (5 ml.) and with water until neutral. The extract was evaporated to dryness below

40° and under N_2 and redissolved in 5% (v/v) ethanol in benzene (4 ml.). Alumina (100 mg., moisture content 2–10%) was added and the solution was shaken for 30 sec. The alumina was allowed to settle and a portion of the solution (3 ml.) taken for determination of 17-oxo steroid by the Zimmermann reaction.

This method measures only steroid glucuronides; the steroids conjugated as sulphate remain in the aqueous diluting fluid after extraction with dichloromethane. The treatment of the 17-oxo steroid extract with alumina is not an essential step in all circumstances but helps by removing material which would interfere with the Zimmermann reaction.

Determination of glucuronic acid. This determination was carried out as a qualitative procedure based upon a method described by Paul (1951) (see Mead, Smith & Williams, 1958).

Determination of 17-oxo steroids. 17-Oxo steroids were determined by the method of Callow *et al.* (1938). The method was reduced to one-quarter scale so that dilution of the final solution was made with 2·5 ml. of ethanol. The optical density was read in a Unicam spectrometer (model SP. 600) at 440, 520 and 620 mμ. Dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one; 10 μg.) was used as standard and Allen's (1950) correction was applied.

Chromatographic analysis of the steroid-conjugate extract. The steroid conjugates from 20 ml. of urine were chromatographed on 5 g. of neutral alumina (M. Woelm, Eschwege; supplier L. Light and Co. Ltd.) having a moisture content adjusted to 5% (loss of weight when heated to 100° for 16 hr.). The column (5 cm. × 1 cm.) was prepared in benzene by allowing the alumina to settle under gravity and then packing tightly with a Martin packer. The slightly pigmented ether–ethanol extract was poured directly on to the column, pigment being adsorbed as a series of bands at the top. The almost colourless filtrate contained less than 5% of the 'total 17-oxo steroids' but contained a large amount of crystalline solid, presumably non-steroid. Any unconjugated steroids present in the extract were found in this fraction.

The column was first washed with absolute ethanol (25 ml.), which eluted more solid material but virtually no 17-oxogenic or 17-oxo steroid.

The eluent was then changed to aqueous 50% (v/v) ethanol (25 ml.), which eluted the 17-oxo steroid sulphates together with some pigment and non-steroid solid. Varying small amounts of 17-oxogenic steroid (1–4%) were also found in this fraction.

Finally, the 17-oxo steroid glucuronides and 95–100% of the 17-oxogenic steroid glucuronides adsorbed on the column were eluted with M-acetate buffer, pH 5·0 (40 ml.). Initially the column ran at a moderate rate but slowed considerably when aqueous eluents were introduced. The results were not adversely affected by the application of pressure (up to 10 cm. Hg) to the top of the column as long as the column was not ruptured when pressure was released to introduce new eluents. The procedure up to the collection of the eluates required approx. 1·5 hr.

The results relate to the analysis of steroid conjugates from 20 ml. of urine but the procedure has been carried out on urine volumes ranging from 10 ml. (3 g. of alumina) to 200 ml. (40 g. of alumina) with proportionate adjustment of the volume of eluting solvents. In practice, an ether–ethanol extract was prepared from 40 ml. of urine and one-half of the extract was analysed for steroid glucuronide and steroid sulphate content by conventional procedures of enzyme hydrolysis and continuous ether extraction at pH 1. The other portion of the extract was poured on to the alumina column, which was developed as already described. The composition of the eluates was then determined by differential hydrolysis as above, and also by the acetylation technique.

RESULTS

Analysis of 17-oxo steroid conjugates

The 17-oxo steroid conjugates and the 17-oxogenic steroid conjugates have been dealt with separately. Table 1 shows the results of a typical chromatographic separation of 17-oxo steroid sulphates from 17-oxo steroid glucuronides. On the left of the table is shown the composition of the unchromatographed urinary extract and each subsequent heading represents a column eluate.

Taken together, the ether–ethanol column filtrate and the subsequent ethanol eluate contained only 1·2% of the 17-oxo steroids placed on the column. The determination of the 17-oxo steroids in these fractions was by hot-acid hydrolysis, no attempt being made to determine the mode of conjugation; any unconjugated 17-oxo steroid would be present in the ether–ethanol filtrate.

Table 1. *Chromatography of 17-oxo steroid conjugates*

Conjugates were extracted from normal male urine (20 ml.) and chromatographically separated on neutral Woelm alumina (5 g.; moisture content 5%). Except for the last row of the table, figures are expressed as μg. of 17-oxo steroid conjugate.

Material	Placed on column	Eluted from column: Ether–ethanol	Ethanol	Aq. 50% ethanol	M-Acetate buffer
Sulphate	68·0	2·6*	1·0*	72·0	3·1
Glucuronide	228·0			4·0	215·0
Acetylatable 17-oxo steroid	—	—	—	3·0	219·0
Glucuronic acid	+	Nil	Nil	Nil	+
Wt. (mg.) of solid matter present in solution	110·2	51·2	13·3	27·8	12·2

* Obtained by hot-acid hydrolysis as described in the text.

The aqueous ethanol fraction was taken to dryness under reduced pressure below 40°, ethanol being added to facilitate evaporation. The residue after incubation with β-glucuronidase gave 4 μg. of benzene-soluble 17-oxo steroid but subsequent hydrolysis by continuous ether extraction at pH 1 liberated 72 μg. of 17-oxo steroid, which corresponded closely to the 17-oxo steroid sulphate (68 μg.) present in the unchromatographed extract.

The M-acetate buffer eluate was diluted with an equal volume of water and incubated with β-glucuronidase. With Ketodase no adjustment of pH is usually necessary but with limpet β-glucuronidase the pH was adjusted to 4·0 and 0·3 M-KH_2PO_4 added. Enzyme hydrolysis liberated 215 μg. of 17-oxo steroid compared with 228 μg. in the parallel extract and subsequently an additional 3·1 μg. was freed after ether hydrolysis at pH 1. No free 17-oxosteroid was extracted from this eluate by benzene before enzyme hydrolysis. The 17-oxo steroid and 17-oxogenic steroid glucuronides can be quantitatively recovered from the acetate buffer eluate by extracting this fraction by the procedure described for urine. The extract obtained in this way from the buffer fraction contained all the steroid glucuronides; on evaporation to dryness it contained between 10 and 15 % of the weight of solid matter placed on the column in the original ether–ethanol extract of urine.

Analysis of 17-oxogenic steroid conjugates

Data relating to the behaviour of 17-oxogenic steroid glucuronides on the same column are given in Table 2. The figures quoted are for total 17-oxo steroids and relate only to material conjugated as glucuronide, for 17-oxogenic steroid sulphates, if excreted, would not be measured by the method used. With this sample 86 % of the 17-oxogenic material present in the urine was extracted by the ether–ethanol procedure. Of the total amount placed on the column 4·5 % was eluated in the ether–ethanol filtrate; unconjugated steroids appear in this fraction and unconjugated cortisone has been identified by paper chromatography as being present. Very little 17-oxogenic steroid was present in the ethanol fraction and only 15 μg. of 'total 17-oxo steroid' glucuronides (3·5 %) was found in the aqueous ethanol eluate.

Substantially all the 17-oxogenic steroid glucuronides were found in the acetate buffer fraction and subsequently hydrolysis with β-glucuronidase liberated 96·5 % of this material. Several attempts were made to determine 17-oxogenic steroid sulphates in the aqueous ethanol and acetate buffer by comparing the figures obtained for 17-oxo steroid sulphates (ether extraction at pH 1) before and after oxidation with sodium bismuthate. The 17-oxo steroids determined before oxidation have been equal to or greater than the value after oxidation and no evidence for the presence of sulphate-bound 17-oxogenic steroid has been found.

Application of the method

The procedure offers a rapid method of determining the ratio of 17-oxo steroid sulphates to glucuronides and, at the same time, supplies sufficient material for differential analysis of the 17-oxo steroids of each fraction. Small portions of the aqueous ethanol and acetate buffer eluates are hydrolysed by the hot-acid procedure and determined in the usual way. A comparison of results obtained by this method with those obtained by the more laborious procedure of enzyme hydrolysis and ether extraction at pH 1 are shown in Table 3.

Table 2. *Chromatography of 17-oxogenic steroid glucuronides*

Conjugates were extracted from normal male urine (20 ml.) and chromatographically separated on neutral Woelm alumina (5 g.; moisture content 5 %). The figures quoted are 'total 17-oxo steroid glucuronides', which include 17-oxo steroid glucuronides; they are given in terms of μg. of 'total 17-oxo steroids' after oxidation with bismuthate.

	Eluted from the column				
Placed on column	Ether–ethanol	Ethanol	Aq. 50 % ethanol	M-Acetate buffer	Hydrolysed by enzyme
443·0	20·0	4·2	15·0	435·1	430·0

Table 3. *Comparison of the glucuronide/sulphate ratio for 17-oxo steroids determined by (a) chromatographic separation followed by hot-acid hydrolysis and (b) β-glucuronidase hydrolysis followed by continuous ether extraction at* pH 1

Results are expressed as μg. of 17-oxo steroid.

	(a)	(b)		
Column fraction	Hot-acid hydrolysis	β-Glucuronidase hydrolysis	Hydrolysis at pH 1	Total
Aq. ethanol	83·0	4·0	72·0	76·0
Acetate buffer	210·0	215·0	3·1	218·1
Ratio glucuronide/sulphate	0·395	—	—	0·349

The results are typical of those obtained in a number of similar determinations on diverse urine samples. The amount of 17-oxo steroid found in the acetate buffer fraction, whether hydrolysed by β-glucuronidase or hot acid, corresponds closely to the amount liberated from urine conjugate extracts by enzyme hydrolysis. Whereas ether hydrolysis of the aqueous–ethanol fraction at pH 1 gives close correspondence with figures obtained by applying the same method to the urine-conjugate extract, hot-acid hydrolysis of this fraction usually gives a higher figure (10–15%). As the difference between the hot-acid hydrolysis and hydrolysis at pH 1 of the aqueous ethanol fraction is not accounted for by the small amount of 17-oxo steroid liberated by β-glucuronidase, these results suggest the presence in this fraction of some type of 17-oxo steroid conjugate other than glucuronide and sulphate.

DISCUSSION

Previous attempts to chromatograph steroid conjugates on alumina have not been encouraging (West, Reich & Samuels, 1951); in spite of the use of very polar organic solvents the recovery of adsorbed material was poor and there was no evidence of separation. As far as 17-oxo steroids are concerned the procedure now described separates sulphates and glucuronides with quantitative recovery. The conjugates obtained in this way show no abnormal behaviour; they are still hydrolysed by the accepted techniques and the liberated 17-oxo steroids behave on paper chromatograms in the normal manner. When separating the conjugates of 17-oxo steroids the moisture content of the alumina is not a critical factor. Neutral alumina with moisture content varying between 0 and 10% has been equally effective.

It is not usually considered practicable to chromatograph corticosteroids on alumina. Reichstein, for his work on adrenocortical extracts, took considerable care to prepare neutral alumina, yet experienced some loss of material (Reichstein & Shoppee, 1949), and there are several reports in the literature of molecular re-arrangements on alumina inside and outside the steroid field. In the present application there was quantitative recovery of adsorbed 17-oxogenic steroid glucuronides in the acetate buffer fraction. That these steroids are still present as glucuronides is shown by quantitative liberation of the free steroids by β-glucuronidase and, furthermore, the side chain of the steroids is still capable of being oxidized by sodium bismuthate to 17-oxo steroid. Paper chromatography of corticosteroids liberated by enzyme hydrolysis from the acetate buffer fraction gives a conventional pattern of urinary-corticoid metabolites. In an application of the procedure (Ayres *et al.* 1957) these products of enzyme hydrolysis were extensively chromatographed on partition columns yet showed no abnormal behaviour.

The results do not establish that 17-oxogenic steroids are invariably excreted in the urine as glucuronides, because the method employed measures only 17-oxogenic steroid glucuronides; nevertheless, attempts made to measure 17-oxogenic sulphates met with no success and it seems unlikely that this form of conjugate represents more than a very small percentage of the total.

Unsuccessful attempts have been made to separate individual glucuronides in the acetate buffer eluate by re-adsorption on alumina, followed by gradient elution. With the sodium acetate–acetic acid buffer system, a gradient of hydrogen-ion concentration at constant molarity and a gradient of molarity at constant hydrogen-ion concentration (pH 5) failed to separate the glucuronides of androsterone (3α-hydroxy-5α-androstan-17-one) and aetiocholanolone (3α-hydroxy-5β-androstan-17-one), yet some buffers are more effective eluting solvents than others; thus quantitative elution of 'total 17-oxo steroid' glucuronides is accomplished by 0·04 M-sodium phosphate–citric acid buffer (pH 5), whereas 0·04 M-sodium acetate–acetic acid buffer (pH 5) eluates approximately 70% of the 'total 17-oxo steroid' glucuronides. Furthermore, 17-oxogenic steroid glucuronides and 11-oxy-17-oxo steroid glucuronides can be eluted quantitatively by 0·1 M-acetic acid, whereas under these conditions most of the 11-deoxy-17-oxo steroid glucuronides remain on the column until sodium acetate is introduced. The use of acetate buffer (pH 5) is preferable to that of phosphate–citrate because β-glucuronidase (Ketodase) can be added directly for enzyme hydrolysis and also because the former eluates less pigment from the column. According to Abul-Fadl (1957) citrate is a mild inhibitor of β-glucuronidase.

SUMMARY

1. A method has been described whereby urine extracts containing steroid conjugates can be chromatographed on neutral alumina.

2. 17-Oxo steroids conjugated as sulphates have been separated from those conjugated as glucuronides; determinations carried out on the fractions so obtained agree with values for these conjugates by alternative methods.

3. 17-Oxogenic steroid glucuronides (Norymberski, 1952) are also recovered quantitatively from the column and neither these nor the 17-oxo steroid conjugates appears to have been modified during adsorption.

4. The procedure provides a rapid method for the determination of 17-oxo steroid sulphate/glucuronide ratios and forms the basis of a method for the partial purification of steroid glucuronides.

5. Attempts to isolate individual conjugates by gradient elution on these columns have not been successful.

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Seasonal Changes of the Catechol Compounds Present in Rat Tissues

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Raab (1941*a*, *b*, 1943*a*), using Shaw's colorimetric method of estimation, reported that the concentrations of 'adrenaline-like catechols' in rat tissues were higher in winter than in summer. This has been confirmed in the present experiments with the ethylenediamine method of Weil-Malherbe & Bone (1953); these experiments have also shown that lower amounts of catechol compounds are present in spring and early autumn (Montagu, 1956*a*).

The fluorescence produced in the ethylenediamine reaction was previously attributed to noradrenaline and adrenaline (Montagu, 1956*b*). Further investigation has shown that a third basic compound is sometimes present (Montagu, 1957). Catechol compounds other than noradrenaline and adrenaline have been found in several animal tissues: examples are 3-hydroxytyramine in sheep heart (Goodall, 1950), in adrenals of sheep and cattle (Shepherd & West, 1953; Holtz & Westermann, 1956) and in phaeochromocytoma (Weil-Malherbe, 1956); *iso*propylnoradrenaline in cat adrenals (Lockett, 1954); 3:4-dihydroxyphenylalanine in ox adrenals (Werle & Jüntgen-Sell, 1955) in phaeochromocytoma (Weil-Malherbe, 1956) and in a human brain (Montagu, 1957). 3:4-Dihydroxyphenylacetic acid is present in urine (Euler, Euler & Floding, 1955; Weil-Malherbe & Bone, 1957).

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METHODS

The experiments date from April, 1954. Male rats of the Wistar strain, isolated from females, were used. They received a standard diet (diet 41; Bruce & Parkes, 1949, 1950) and drinking water *ad lib.* They were housed in a separate building 16 yards from the Laboratory, exposed to daylight and kept, from October until May, at 65–70° F. They were killed in the morning by a blow on the head within 15 min. of being brought in from the animal house.

Estimation of 'total catechols'. The heart, brain, spleen, diaphragm muscle, both kidneys and part of the liver and of the leg muscles of a single rat were separately extracted and treated as already described (Montagu, 1956*b*). The fluorescence in the final solution was measured with yellow and blue–green filters after condensation with ethylenediamine dihydrochloride (ED). As catechol compounds other than adrenaline and noradrenaline may be present, the calculation previously used may not be valid. The fluorescence produced by the mixture of catechol compounds was therefore compared with that of a standard solution of adrenaline, and the concentration of 'total catechols' calculated in terms of this compound.

During the 3½ years' period of this investigation three modifications in the method were introduced: (1) in April

1955 the fluorimeter was modified as described by Weil-Malherbe & Bone (1957); (2) in November 1955 the phosphate solution used for the partial neutralization of the crude tissue extracts was replaced by 1 ml. of 1% (w/v) ascorbic acid and 5 ml. of a solution containing 2% (w/v) of $Na_2S_2O_3.5H_2O$ and 1% of disodium ethylenediaminetetraacetate (pH 7·2); (3) from May 1956 estimation of the separate catecholamines was also carried out and larger amounts of tissue were homogenized in 4 vol. of acid methanol and re-extracted once instead of twice with a further 2 vol. For liver, the extracts were acidified to pH 3 with 5N-HCl, and half or more of the methanol was then removed by distillation *in vacuo* at 37° to reduce the amounts of *n*-butanol and HCl required in the subsequent extraction. After the tissue extracts had been filtered through alumina, 'total catechols' were estimated in a sample of the eluate and the rest was analysed further as described below.

Estimation of catecholamines. Basic compounds were separated from neutral and acidic components by treatment with the cation-exchange resin Amberlite IRC-50 (British Drug Houses Ltd., analytical grade, 16–50 mesh) buffered at pH 6·5, the procedure following approximately that of Bergström & Hansson (1951). The moist resin (2 g.) in a column of 6 mm. bore was prepared for use by treating it with 25 ml. quantities of N-HCl, water, N-NaOH, water, 0·5M-phosphate buffer (pH 6·5) and water at a filtration rate not exceeding 1 ml./min. Recycling was continued until the HCl filtrate gave no fluorescence on treatment with ED. The tissue extract was then mixed with 0·1 vol. of 1% disodium ethylenediaminetetraacetate, and adjusted to pH 6·5±0·1 and filtered through Whatman no. 5 paper to remove traces of alumina; it was poured on to the column of resin, the column was washed with 25 ml. of 0·2% NaCl and the alkaline compounds were eluted with 30 ml. of N-HCl at a rate not exceeding 0·8 ml./min. After recycling once, the column was ready for use again. A part of the eluate, magnetically stirred, was adjusted to pH 3·5 by dropwise addition of N-NaOH, and was used for estimation of the total basic fraction by the ED method, and of noradrenaline and adrenaline by the trihydroxyindole (THI) method of Euler & Floding (1955). In this last method all solutions, including standards of adrenaline and noradrenaline, were diluted to 5 ml. with approx. 0·3 mN-HCl before estimation. The rest of the procedure was as described by Weil-Malherbe & Bone (1957) except that the alkaline ascorbate contained only 0·72 ml. of 2% (w/v) ascorbic acid and the final volumes were not made up to 10 ml.

The estimates of noradrenaline and adrenaline were often insufficient to account for the fluorescence produced in the ED method. The concentration of a third compound, X, which is probably hydroxytyramine (Montagu, 1957), was calculated in terms of this compound as follows: let a, n and h denote the ED fluorescence (yellow filter) produced by 200 μmg. of pure adrenaline, noradrenaline and hydroxytyramine respectively, and let x denote the ED fluorescence produced by the eluate sample. If A and N are the amounts, in μmg., of adrenaline and noradrenaline found in an identical sample by the THI method, the amount of hydroxytyramine is $(200x - aA - nN)/h$ μmg.

Estimates of catecholamines were not corrected for the oxidation of between 4 and 15% of the noradrenaline which occurred at pH 3·5 in the THI method. There was no seasonal variation in the extent of this oxidation. Correction would have increased noradrenaline and reduced adrenaline estimates by about 9·5% and about 4% respectively of the noradrenaline concentrations shown in the results. The latter factor is relatively large. The effects on X would be negligible. The ascorbic acid added in the THI method produced slight fluorescence, but the reagent blank was not corrected for this.

Statistical analyses. The significance of the estimated amounts of catechol compounds and of their seasonal and other changes has been investigated by Student's *t*-test (McNemar, 1949). Occasionally the estimated amount of basic catechol compounds was less than that of the sum of noradrenaline and adrenaline, leading to a negative estimate of the concentration of X. Such negative estimates are clearly due to experimental error and have been included for statistical analysis.

Operations. Bilateral adrenalectomy or bilateral adrenal demedullation was performed in one stage with ether–air anaesthesia. The rats were maintained on the standard diet with 1% NaCl solution instead of drinking water. Water was restored to demedullated rats 2–3 weeks after operation. Demedullation was accomplished by expressing the contents of the adrenal capsule; the cortex regenerates within a month (Ingle & Griffith, 1949), as was shown by a normal insulin tolerance and by histological examination of representative sections from eight glands. A trace of chromaffin tissue was found in one. The insulin tolerance of rats adrenalectomized 2–3 weeks previously was about one-hundredth of that of normal rats. Rats which had received insulin were not used in the experiments reported below.

RESULTS

3-Hydroxytyramine, *iso*propylnoradrenaline, 3:4-dihydroxyphenylalanine (DOPA) and 3:4-dihydroxyphenylacetic acid (DOPAC) are all adsorbed by alumina at pH 8·4 and form fluorescent compounds in the ED reaction. The fluorescence intensities of these compounds relative to that of the adrenaline derivative are shown in Table 1. In the *iso*butanol phase the ratio of the fluorescence measured with the blue–green filter to that with the yellow filter is unity with adrenaline and *iso*propylnoradrenaline, but is greater with each of the other compounds. With DOPA and DOPAC, unlike the basic compounds, the fluorescence in the *iso*butanol phase is less than that in the aqueous phase.

The concentrations of 'total catechols' in tissues have been measured with yellow and blue–green filters and are expressed accordingly. The ratio of the blue–green to the yellow fluorescence was not constant, showing that the proportion of the various components varies, and that the calculated amounts expressed in terms of adrenaline have only qualitative significance. The amounts of total catechols varied significantly from season to season whether comparison was made between all the results obtained in each month during the whole period of study, or whether the results of each year were considered separately. Concentrations in each

Table 1. *Fluorescence intensities of equimolecular amounts of various catechol compounds after condensation with ethylenediamine*

Intensities are expressed relative to those of adrenaline, arbitrarily taken as 100 with each filter in the *iso*butanol phase. Examples of the fluorescence in extracts of equal weights of rat heart and brain, eluted from alumina and condensed with ED, are included.

Compound or tissue	*iso*Butanol phase: With yellow filter (*Y*)	*iso*Butanol phase: With blue–green filter (*B*)	Ratio (*B*/*Y*)	Aqueous phase with yellow filter
Adrenaline	100	100	1·0	2
Noradrenaline	43	145	3·4	2
3-Hydroxytyramine	38	67	1·8	2
*iso*Propylnoradrenaline	52	52	1·0	2
3:4-Dihydroxyphenylalanine	0·9	1·8	2·0	5
3:4-Dihydroxyphenylacetic acid	6·8	25	3·7	11
Rat heart	40	122	3·1	3
Rat brain	62	149	2·4	4

Table 2. *Concentrations of 'total catechols' in heart estimated by the ethylenediamine method with the yellow filter*

Results are expressed as μmg./g. of heart (wet wt.)±S.E. Black figures denote the numbers of estimations.

	April	May	June	July	August	September
April 1954–March 1955	41·8 1	239±46·3 4	191±22·3 4	443±66·7 7	262±78·0 5	264±61·6 6
April 1955–March 1956	—	346 1	—	—	—	—
April 1956–March 1957	—	168 1	206±46·6 4	234±12·0 2	228 1	243±35·0 2
April 1957–August 1957	211±30·3 5	283±16·5 3	324±14·6 2	—	173±52·0 2	—
	October	**November**	**December**	**January**	**February**	**March**
April 1954–March 1955	396±46·9 11	451±147 4	502±35·7 13	—	398±2·0 2	169±14·5 2
April 1955–March 1956	— —	603±269 7	1690 1	691±164 4	—	220±80·9 5
April 1956–March 1957	334 1	446±41·3 3	535±175 2	319±21·1 3	—	212±15·7 3
April 1957–August 1957	—	—	—	—	—	—

The significance (*P*) of differences of concentration in several groups of months, denoted by Roman numerals. The low concentrations found in spring and autumn were combined and tested for significance against the higher concentrations in summer and in winter.

		Summer	Winter
April 1954–March 1955	(IV+VIII+IX) 1954+(III) 1955	VII; $P=0.02$	XI+XII+I; $P<0.001$
April 1955–March 1956	(V) 1955+(III+V+VI) 1956	(No estimations)	XI+XII+I; $P<0.02$
April 1956–March 1957	(IV+VIII+IX) 1956+(III) 1957	VII, or V to VII, not significant	XI+XII+I; $P<0.01$
April 1957–August 1957	(III+IV+VIII) 1957	V to VII; $P<0.01$	(No estimations)
April 1954–August 1957	(III+IV+VIII+IX)	VII; $P<0.01$	XI+XII+I; $P<0.001$

month are shown in Tables 2 and 3. They were lowest in March to April and in August and September, and were highest in the winter (December, January or February). Increases also occurred during the summer (May to July), but were smaller except in liver. The changes of concentration in the seven tissues have been combined in Fig. 1; this shows that the ratio of the fluorescence with the blue–green filter to that with the yellow was unusually low in February and July.

Compounds producing the ethylenediamine fluorescence in tissue extracts. Catecholamines may be separated from acidic and neutral compounds by adsorption of the catecholamines on Amberlite IRC-50 resin at pH 6·5. On elution with acid the ED fluorescence of the eluate was 85–100 % of its value before treatment with resin, and the ratio of yellow to blue–green fluorescence was unchanged. Pure solutions of adrenaline and noradrenaline gave recoveries of 90–100 %. Before treatment with the resin the fluorescence in the aqueous phase was variable but small, and was not significantly changed after the removal of any non-basic compounds, such as DOPA or DOPAC, which might have been present. There seems to be no evidence for the presence of these compounds in the tissue extracts. DOPA forms a fluorescent derivative in the THI method (Weil-Malherbe & Bone, 1957), and the fluorescence is greater after oxidation at pH 6·0 than at pH 3·5. The filtrates from resin columns were equally fluorescent after oxidation at the lower pH.

Catecholamines. The amounts of noradrenaline and adrenaline estimated by the THI method in the acid eluates from the resin were usually insufficient to account for the estimates of the total basic fraction; this is shown in Tables 4 and 5, in which the difference, compound X, has been expressed in terms of hydroxytyramine. Table 5 shows the concentrations in heart and brain in different months; it suggests that there are probably seasonal changes of the concentrations of each of the three catecholamines and of their combined amounts.

The concentration of X was highest in brain and must account for much of the adrenaline previously reported in that tissue (Montagu, 1956*b*). It was

Table 3. *'Total catechols' in various rat tissues estimated in different months by the ethylenediamine method*

The yellow filter was used and the results are expressed in μmg. of adrenaline/g. wet wt. of tissue, except for spleen (μmg. of adrenaline/organ). Figures in parentheses indicate the corresponding results obtained with the blue–green filter. Black numbers denote the numbers of estimations.

	April	May	June	July	August	September
Brain	319 (609)	411 (714)	370 (923)	252 (449)	290 (726)	—
	5	1	4	2	2	0
Heart	183 (479)	258 (722)	224 (707)	397 (764)	236 (545)	258 (569)
	6	9	10	9	8	10
Spleen	125 (375)	—	151 (556)	122 (302)	166 (292)	137 (281)
	7	0	4	2	2	2
Diaphragm	85·3 (228)	88·0 (126)	62·7 (279)	153 (357)	74·7 (175)	122 (284)
	4	1	5	7	3	7
Kidneys	29·6 (66·6)	24·4 (56·4)	54·3 (117)	104 (189)	82·7 (207)	60·4 (111)
	4	1	3	4	6	3
Liver	21·3 (58·3)	49·4 (145)	47·6 (108)	70·6 (124)	41·9 (112)	46·5 (106)
	15	6	8	10	3	8
Leg muscles	13·6 (44·9)	31·2 (65·7)	17·6 (34·5)	48·1 (102)	16·0 (35·1)	23·0 (47·4)
	2	3	3	4	2	7
	October	November	December	January	February	March
Brain	—	480 (1040)	532 (1120)	640 (1350)	584 (982)	391 (731)
	0	9	4	4	2	10
Heart	390 (971)	534 (1340)	580 (1510)	531 (1670)	308 (1020)	208 (498)
	12	13	17	7	2	10
Spleen	—	144 (435)	192 (671)	327 (1280)	—	114 (234)
	0	7	3	5	0	6
Diaphragm	—	141 (390)	288 (1020)	198 (741)	219 (252)	67·9 (209)
	0	6	4	12	1	6
Kidneys	113 (296)	148 (434)	156 (455)	144 (468)	153 (303)	60·0 (129)
	10	6	18	5	2	7
Liver	—	51·8 (137)	45·0 (113)	65·7 (190)	79·9 (185)	24·1 (64·4)
	0	17	5	12	3	11
Leg muscles	—	33·2 (77·3)	33·2 (81·2)	43·2 (115)	44·9 (90·8)	16·5 (77·9)
	0	4	1	7	1	1

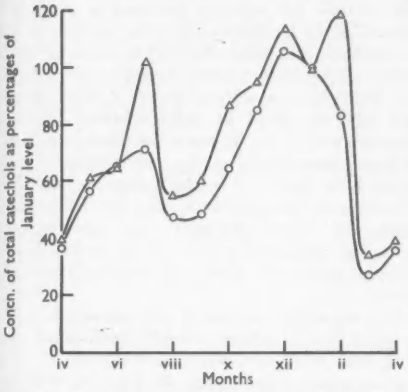

Fig. 1. Seasonal changes of the concentrations of total catechols, estimated by the ethylenediamine method with yellow (△) and blue–green (○) filters, in rat heart, brain, spleen, kidney, diaphragm, liver and leg muscles. Considering each tissue separately, the concentrations for each month in the different years were calculated as percentages of those in January of the following year, and were denoted by m. A loaded mean for all tissues in each month was then calculated from the formula

$$\overline{m} = \frac{\Sigma(\rho m)}{\Sigma \rho},$$

where ρ was either the number of estimates in January, or the number of estimates in the particular month in question, whichever was the smaller.

also found in liver, but only sometimes in spleen, heart, kidneys and diaphragm. The mean concentrations found in heart in each of the months March, April, June, August and September, and the mean concentration in diaphragms, were not statistically significant.

Comparison of normal, adrenalectomized and demedullated rats. Some experiments were carried out to investigate the possible dependence of the seasonal changes on the activity of the adrenal medulla or cortex. Rats were bilaterally adrenalectomized in September–October, when concentrations of 'total catechols' are increasing, and in January–February when they are decreasing; they were killed some weeks later at the same time as control rats and the hearts were analysed. It was found that when adrenalectomy had been performed in the autumn the amounts of total catechols 6–10 weeks later were lower in the adrenalectomized rats than in their controls, but that the reverse was the case when the operation was performed in January–February (Table 6, *a–b*). These two effects of adrenalectomy were statistically different whether the results were expressed as concentrations or as μmg./whole heart. The results in Table 6 have been expressed as μmg./organ, as heart weights were significantly higher ($P < 0.01$) in the adrenalectomized rats; the weights of these rats were similar to those of their controls at operation and *post mortem*. Table 6 also shows the changes which were found in the amounts of the individual catecholamines. The table suggests that seasonal changes of catechol compounds in heart are reduced by adrenalectomy.

Rats adrenalectomized only 11 days previously were compared with the control rats used in some of the above experiments (Table 6, *c–d*). Here also the effects of operation depended on the time of year at which it was carried out, and the changes of total catechols produced by adrenalectomy in November were probably significantly different from those produced in March. They were smaller than in rats adrenalectomized less recently and differences of heart weight were not detected. In five experiments cortisone acetate (3 mg. intraperitoneally for 3 days before killing) had no significant effects on the amounts of total catechols or of catecholamines in hearts of rats adrenalectomized 11 days before analysis in November.

In some of the above experiments brains were also analysed. No significant effects of adrenalectomy were found (Table 6, *e–g*).

In other experiments rats were bilaterally demedullated in September, when concentrations of catechol compounds are low, and were compared with control rats 5–7 weeks later (Table 7). Demedullation had not significantly altered the concentration of total catechols in heart, but had increased ($P < 0.01$) the ratio of the ED fluorescence with blue–green and yellow filters. The total catechols in kidneys of demedullated rats were lower than in the control rats analysed simultaneously, but were probably higher than in intact rats analysed at the time of operation.

DISCUSSION

The concentrations of total catechols, estimated by the ethylenediamine method in seven rat tissues, increase more extensively in the winter than can be accounted for by the minor changes of tissue weight which may occur (see Raab, 1941*a*, *b*). Non-basic compounds contribute little if at all to the ED estimates of total catechols, and the seasonal changes are therefore changes of basic compounds. The available evidence (see Fig. 1 and Tables 1 and 5) suggests that the concentrations of noradrenaline, adrenaline and of X increase progressively from September to December, with some conversion of X into noradrenaline in January and of noradrenaline into adrenaline in February. Fig. 1 also suggests that a similar sequence may occur between April and July; summer increases are smaller and less easily established.

Experiments were carried out to investigate the possible relation to the adrenal glands of the seasonal changes occurring between autumn and spring. The amounts of total catechols in heart appeared to increase after removal of the adrenals in the autumn, and to decrease when adrenalectomy was performed in January. The changes were probably smaller than the seasonal changes occurring in normal rats and suggest a partial dependence of these changes on the adrenal gland. It is also possible that after adrenalectomy the concentrations of total catechols find an intermediate level which shows no seasonal change. Experiments with rats which had been adrenalectomized only 11 days previously did not eliminate either possibility.

When rats which had been demedullated in September were killed in November, the concentrations of total catechols in hearts were not different from those in control rats analysed simultaneously; this was in spite of the temporary removal of cortex involved in the process of demedullation. Since the autumnal increase of total catechols seems therefore to occur in the absence of the medulla the ensuing decrease is unlikely to depend on its presence. An alteration in the demedullated rats of the ratio of the ED fluorescence determined with two filters suggests a reduction of the proportion of adrenaline or X, but this was probably an effect of demedullation only. In contrast with those in heart the total catechols in kidneys were reduced by demedullation; this might have been due to the operation itself or to reduction or prevention of seasonal changes. In brain, an organ generally less susceptible to hormonal influence, there was no evidence that seasonal differences involve the adrenal medulla or cortex.

The seasonal changes of the concentrations of catechol compounds in the different tissues might arise from intrinsic variations of any or all of the various internal secretions. Weather and duration of daylight seem to be the other possibilities; owing to increases of catechol compounds in summer as well as winter neither alone is likely to provide adequate explanation. However, the external temperature was lower, and the estimates of total catechols higher, in the winter of 1955–56 than in

Table 4. *Noradrenaline, adrenaline and compound X in rat tissues*

Results are expressed for spleen in μmg./whole spleen, and for diaphragm, kidney and liver in μmg./g. wet wt. Compound X is calculated in terms of hydroxytyramine. Results were obtained in June, July, December, January and April and are listed as means ± S.E.

	Spleen	Diaphragm	Kidney	Liver
Noradrenaline	144 ± 21·0	86·5 ± 12·5	72·8 ± 11·7	28·6 ± 7·55
Adrenaline	40·3 ± 6·90	34·0 ± 5·51	19·9 ± 3·10	9·07 ± 4·32
Compound X	110 ± 33·8	36·5 ± 20·6	21·2 ± 6·11	18·9 ± 6·45
No. of estimates	7	8	10	11

Table 5. *Concentrations of noradrenaline, adrenaline and compound X in rat heart and brain*

Concentrations are expressed as μmg./g. of tissue (wet wt.) and months are denoted by Roman numerals. Compound X is calculated in terms of hydroxytyramine.

	VI	VII	VIII	IX	XI	XII	I	II	III	IV	V
Heart											
Noradrenaline	230	288	203	168	400	—	439	—	270	248	326
Adrenaline	38·7	23·0	57·6	99·3	109	—	79·0	—	109	58·3	70·2
Compound X	52·2	132	−65·4	102	232	—	90·0	—	40·5	41·5	84·0
No. of estimations	5	2	1	4	2	0	2	0	3	4	3
Brain											
Noradrenaline	113	143	132	—	120	190	174	260	198	137	250
Adrenaline	20·9	30·0	15·2	—	75·6	93·2	39·6	72·6	53·0	29·9	35·2
Compound X	427	327	378	—	420	439	835	642	408	332	353
No. of estimations	4	2	2	0	2	3	1	2	4	2	1

Estimates of the significance (P) of differences of concentration in groups of several different months

Heart		
Noradrenaline	XI to I > III + IV + VI + VIII + IX	$P < 0{\cdot}01$
Adrenaline	IX to III > IV to VIII	$P = 0{\cdot}001$
Compound X	XI to I > III + IV + VI + VIII + IX	$P < 0{\cdot}01$
Brain		
Noradrenaline	XII to III > IV + (VI to VIII) + XI	$P < 0{\cdot}001$
Adrenaline	XI to II > IV to VIII	$P < 0{\cdot}001$
Compound X	XII to II > IV + V + VII + VIII	$P = 0{\cdot}05$

Table 6. *Effects of adrenalectomy, obtained from simultaneous analysis of adrenalectomized and control rats, and the changes occurring after operation as judged from comparison with intact rats analysed at the times of operation*

Table 6. *Effects of adrenalectomy, obtained from simultaneous analysis of adrenalectomized and control rats, and the changes occurring after operation as judged from comparison with intact rats analysed at the times of operation*

Pooled rats were used in each experiment. Changes and percentage changes of the amounts, in μmg./whole organ, of total catechols, estimated by the ethylenediamine method with yellow and blue–green filters (ED_Y and ED_B), and of noradrenaline (N), adrenaline (A) and of compound X, are shown. The significance *P* of the effects of adrenalectomy was assessed from *t* tests of the differences, expressed as percentages, between adrenalectomized and control rats, except for compound X where *P* was calculated from actual differences. Numbers of experiments listed under 'changes since operation' refer to numbers of analyses made at the times of operation. Time of operation, calculated as a mean date, and of subsequent analysis, is included.

	Effect of adrenalectomy					Change since adrenalectomy		Amounts in adrenalectomized rats (μmg./organ)					Rat wt. (g.)		Tissue wt. (g.)
	ED_Y	ED_B	N	A	X	ED_Y	ED_B	ED_Y	ED_B	N	A	X	At operation	*Post mortem*	
Heart															
(*a*) Date of operation 3. x. 56; date of analysis 16. xi. 56															
Change	−99·7	−217	+12·5	−36·4	−78·5	+61·0	+180	227	634	226	31·6	147	C.* 154	225	0·658
% change	−30	−26	+1·0	−53	—	+37	+40	—	—	—	—	—	O.* 163	219	0·723
P	<0·01	<0·05	>0·2	<0·05	>0·2										
No. of expts.	3	3	2	2	2	3	3	5	5	2	2	2			
(*b*) Date of operation 30. i. 57; date of analysis 27. iii. 57															
Change	+45·0	+128	+44·3	−10·9	+125	−16·0	−149	214	613	229	72·6	167	C. 159	277	0·741
% change	+25	+29	+31	−12	—	−7·0	−0·20	—	—	—	—	—	O. 157	276	0·910
P	<0·05	<0·05	>0·2	<0·1	<0·1										
No. of expts.	5	5	4	4	4	3	3	5	5	4	4	4			
(*c*) Date of operation 17. xi. 56; date of analysis 29. xi. 56															
Change	−35·6	−106	−42·6	+5·8	−73·5	—	—	166	469	166	68·7	93·7	C. 170	180	0·576
% change	−17	−19	−23	+11	—	—	—	—	—	—	—	—	O. 168	182	0·587
P	<0·02	<0·02	<0·02	>0·2	<0·2										
No. of expts.	5	5	5	5	5	—	—	5	5	5	5	5			
(*d*) Date of operation 15. iii. 57; date of analysis 26. iii .57															
Change	−10·3	−33·3	+12·5	−15·5	+36·7	—	—	164	487	178	70·9	88·7	C. 237	261	0·777
% change	−6·0	−5·2	+29	−18	—	—	—	—	—	—	—	—	O. 240	249	0·716
P	<0·05	>0·2	>0·2	=0·05	<0·1										
No. of expts.	3	3	3	3	3	—	—	3	3	3	3	3			
Brain															
(*e*) Date of operation 18. x. 56; date of analysis 11. xii. 56															
Change	+1·0	−60·0	+46·0	−62·0	+348	—	—	705	1670	298	135	605	C. 142	245	1·51
% change	+0·1	−3·5	+18	−31	—	—	—	1	1	1	1	1	O. 139	238	1·49
1 expt.															
(*f*) Date of operation 30. i. 57; date of analysis 27. iii. 57															
Change	+3·2	+44·2	−11·8	+4·2	+107	−287	−428	483	1080	261	86·1	684	C. 178	275	1·48
% change	+0·4	+2·7	−2·6	+11	—	−37	−28	—	—	—	—	—	O. 168	264	1·53
P	>0·2	>0·2	>0·2	>0·2	=0·2										
No. of expts.	5	5	5	5	5	2	2	5	5	5	5	5			
(*g*) Date of operation 15. iii. 57; date of analysis 26. iii. 57															
Change	−7·0	−99·0	—	—	—	—	—	427	834	—	—	—	C. 242	261	1·48
% change	−0·9	−11	—	—	—	—	—	—	—	—	—	—	O. 240	249	1·57
P	>0·2	<0·1													
No. of expts.	3	3	—	—	—	—	—	3	3	—	—	—			

* C. = control; O. = operated.

Table 7. *Effects of bilateral adrenal demedullation on the concentrations of total catechols in heart and kidneys, and the changes occurring after operation*

Single rats were compared on separate occasions. Concentrations of total catechols were estimated in μmg./g. of tissue (wet wt.) by the ethylenediamine method with yellow and blue–green filters (ED_Y and ED_B); *t* tests were carried out on differences between demedullated and intact rats.

		Effect of operation		Change since operation		Concentrations in operated rats		Rat wt. (g.)		
		ED_Y	ED_B	ED_Y	ED_B	ED_Y	ED_B	At operation	*Post mortem*	Tissue wt. (g.)
Date of operation 18. ix. 54; date of analysis 24. x. 54										
Heart	Change	+34·0	+165	+114	+461	437	1140	C.* 205	274	0·811
	% change	+8·4	+17	+35	+68	—	—	O.* 194	257	0·760
	P	>0·2	<0·2	<0·05	<0·01					
	No. of expts.	10	10	7	7					
Date of operation 18. ix. 54; date of analysis 24. x. 54										
Kidney	Change	−26·0	−74·0	+29·2	+113	91·0	228	C. 205	274	1·92
	% change	−27	−25	+47	+98	—	—	O. 194	257	1·87
	P	<0·01	<0·01	<0·05	<0·05					
	No. of expts.	10	10	3	3					

* C. = control; O. = operated.

the preceding or following years. The rats were out-of-doors for less than half a minute shortly before being killed. If the large increases of catechol compounds in heart, which are apparently independent of the adrenal medulla but dependent on the cortex, are due to a brief lowering of temperature, they must be brought about by a mechanism such as, for example, presumably exists for the rapid synthesis or release of catecholamines from adrenergic neurones. It is possible that the generalized sympathetic discharges which result from stimulation of the heat-conserving centre in the hypothalamus (Keller, 1938) depend for their elicitation or effect on the adrenal cortex; it is known that the cortex responds to a reduction of temperature in the absence of circulating adrenaline (Vogt, 1952). Such an explanation would not be enough: the mean temperature in March (42° F.), still well below that in the animal house, is similar to that of November (Manley, 1952); in March the total catechols are at their lowest levels and would probably have been higher had the rats been adrenalectomized early in February. Although varying lengths of days might be associated with changes in the activity of the adrenal glands, it is difficult to attribute the biannual changes of catechol compounds to two environmental factors alone.

The variations of the concentrations of catechol compounds might explain some seasonal changes of metabolism which have been reported. Among these are changes of respiratory quotient (Cori & Cori, 1926) and heat production (Benedict & Macleod, 1929; Sherwood, 1936), of acid production by the gastric mucosa (Mizell, 1955) and of blood lactate (Swan, 1943), seasonal differences in the response to ouabain (Wollenberger & Karsh, 1951) and barbiturates (de Beer, Hjort & Cook, 1939; Weil-Malherbe, 1955), in the ease of falling asleep (Kleitman, 1939; Renton & Weil-Malherbe, 1956) and in the incidence of angina pectoris (Raab, 1943*b*). That few of these are noted as biannual may be due to the usual method of comparing only winter with summer levels.

SUMMARY

1. The combined concentrations of the catechol compounds in heart, brain, spleen, kidney, liver, diaphragm and leg muscles of rats have been estimated by the ethylenediamine method for periods of 1–3½ years; there were large and significant seasonal changes in each tissue; concentrations were highest in winter and summer.

2. Besides adrenaline and noradrenaline, a third catecholamine is present in brain and liver, and sometimes in spleen, heart, kidneys and diaphragm; concentrations of all three catecholamines probably vary seasonally.

3. Adrenalectomy reduced or abolished seasonal changes in heart, but not those in brain. Changes in heart were unaffected by removal of the adrenal medulla; concentrations of catechol compounds in kidney were reduced.

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The Relationship of Fermentation to Cell Structure in Yeast

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From a biochemical point of view, the sequence of reactions in fermentation and glycolysis is well understood, and many of the individual enzymes have been purified and extensively characterized. However, from a cytological point of view, the relationship of fermentation to cellular structure is far from clear. Certain experiments suggest that the glycolysis reactions are located in the periphery of the cell (Rothstein, 1954*a*). For example, in the red blood cell, the uptake of ^{32}P-labelled phosphate apparently proceeds in part via esterification into glycolytic intermediates in the stroma (Gourley, 1952; Prankerd & Altman, 1954). In muscle, radioautographic studies indicate that ^{32}P-labelled phosphate is esterified in the periphery of the cell (Causey & Harris, 1951). In yeast cells, results of detailed studies of sugar uptake have also pointed in the same direction (Rothstein, 1954*b*).

Studies with certain cell-free preparations of yeast suggest a relationship between glycolytic processes and cellular structure. There are basically two types of yeast preparation: the structureless, soluble fermenting system, and the dried-yeast preparations, in which much of the enzyme activity is associated with insoluble cellular residues. In the soluble fermenting systems (press-juice, Lebedev maceration juice, extracts from frozen and thawed cells, and yeast treated by sonic oscillation) certain characteristic properties are found which were described by Harden (1932). Such preparations show a dependence on orthophosphate and possess a characteristic sequence of production of carbon dioxide. In the absence of phosphate, the course of

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fermentation of yeast juice with glucose shows a slight preliminary acceleration, due to free phosphate in the juice, after which there is a steady rate of production of carbon dioxide, which continues for a considerable period. In the presence of phosphate, the rate rapidly rises to a value several times that of the normal rate, then gradually returns to the steady rate obtained with the extract in the absence of phosphate. The extracts can ferment hexose diphosphate at a rate equal to that for glucose. In dried-yeast preparations, however, the fermentation is much more like that of the intact cell, with no Harden–Young sequence, less dependence on orthophosphate and lower rates of fermentation of hexose diphosphate (Nilsson & Alm, 1941; Meyerhof & Wilson, 1949). Meyerhof (1945) has demonstrated that the determining factor in the behaviour of the dried preparation is the preservation of apyrase activity. Its inhibition or destruction results in the appearance of the typical Harden–Young sequence and phosphate-dependence.

The similarity between the fermentation pattern of the dried-yeast preparations and that of intact cells suggested that the dehydration–rehydration process used in the preparation of the dried yeast constituted a minimal disturbance of a cellular structure which carried the glycolytic enzymes. On this premise, the fermentation properties of several dried-yeast preparations were determined. This paper describes the detailed study of one type of preparation whose fermentation and respiration, phosphate uptake and permeability possessed interesting properties. Evidence is presented that the glycolysis reactions are indeed associated with a cellular structure.

METHODS

Yeast preparations

Baker's yeast (Standard Brands Inc., Buffalo, N.Y., U.S.A.) was thoroughly washed, spread in a thin layer (about $\frac{1}{4}$ in. thick) and slowly dried at 22°. In drying, it passes through a crumbly stage, when it can be broken into small granules by hand-kneading. After 24 hr., the yeast material was in the form of a granular dry powder which was lyophilized to a constant dry weight (21% of the wet wt.). The lyophilized yeast was then ground to a fine powder in a Wiley laboratory rotatory mill until it would pass a 50-mesh sieve. The dry preparation was extracted with 10 vol. of acetone and air-dried. It was then treated and washed in a variety of procedures described below. Plating of the preparation on Sabouraud medium revealed no viable cells capable of forming colonies.

Analytical methods

Orthophosphate. This was determined by the method of Fiske & Subbarow (1925) modified by the addition of 15% ethanol before colour development (Meyerhof & Oesper, 1947).

Total phosphate. This was estimated as orthophosphate after hydrolysis of organic phosphates with H_2SO_4.

Potassium. This was estimated by means of the flame photometer (Beckman attachment with a model DU Beckman spectrophotometer).

Glucose. This was determined by the method of Nelson (1944).

Glycogen. This was estimated by the method of Scott & Melvin (1953).

Protein. A volume (4 ml.) of biuret reagent (300 ml. containing 1 g. of $CuSO_4$ and 75 g. of carbonate-free NaOH) was added to 2 ml. of supernatant, then diluted to a total of 10 ml. The colour was read in a Lumetron colorimeter (Photovolt Corp., New York, U.S.A.) with a 525 $\mu\mu$ filter and calibrated against egg albumin.

Ethanol. This was identified by the formation of a derivative with *p*-nitrobenzoyl chloride (Schriner & Fuson, 1940), which was used in the determination of a mixed melting point after microdistillation as described by Gettler, Niederl & Benedetti-Pichler (1932). The estimation of ethanol was carried out by the chromate method (Westerfield, Stotz & Berg, 1942).

Hexose diphosphate. This was estimated as the fructose moiety by the method of Roe, Epstein & Goldstein (1949).

Pyruvate. This was estimated by the method suggested by Umbreit, Burris & Stauffer (1949).

Carbon dioxide and oxygen. These were estimated by the standard Warburg techniques.

Paper chromatography of phosphate compounds. These were separated two-dimensionally on Schleicher and Schuell 589 orange ribbon paper. The solvents used were trichloroacetic acid–acetone at 4° (Burrows, Grylls & Harrison, 1952) and butyric acid–NaOH at 25° (Wade & Morgan, 1955). The presence of the various radioactive compounds on the chromatogram was detected by radioautography with Kodak no-screen X-ray film. The method of Berg (1958) was used for the chemical determination of the position of esters on the chromatogram. The radioactive spots were identified by cochromatography with known pure phosphate compounds.

Measurement of radioactivity. The activity of ^{32}P in the supernatant was determined with 1 ml. of solution by using an end-window Geiger–Müller counter.

Buffer solutions. The buffer used throughout all the experiments was an equimolar mixture of succinic and tartaric acids adjusted to the required pH with pure triethylamine. The final concentration of each acid in the buffer solution was 0·01 M.

Treatment of the dried yeast. The dried yeast, unless it is stated otherwise, was directly suspended in water to produce a final concentration of between 10 and 20 mg./ml. For any one chemical determination, 5 ml. of suspension was removed at the appropriate time and centrifuged. 2 ml. of the supernatant was removed for analysis of the medium. It was appropriately diluted according to the analytical method. In some experiments involving changes in the phosphate content of the medium, precipitation of protein proved to be necessary. A volume (2 ml.) of the supernatant was treated with 5 ml. of trichloroacetic acid (final concentration 10%), and centrifuged and the protein-free supernatant appropriately diluted. This treatment was unnecessary in experiments with washed yeast.

The residue from the above centrifuging, after washing and resuspension in a suitable volume of water, was used for the chemical analysis of the dried yeast. When the orthophosphate content of the dried yeast was determined, the residue was directly extracted twice with 5 ml. of 10% trichloroacetic acid and the extract made up to a suitable volume for analysis. For total phosphate and potassium, 1 ml. of the dried-yeast suspension was heated under reflux with 0·4 ml. of 10N-H_2SO_4 for 15–17 hr. on a sand bath. The temperature of the sand bath was adjusted so that there was gentle refluxing and little liquid lost. The acid-treated material was then boiled with 30% H_2O_2, which was added slowly a drop at a time until the solution was clear (usually, about 1 ml. was added). The solution was analysed as described above. It was necessary for an analysis of the glycogen content to extract 1 ml. of the suspension with 10 ml. of 10% trichloroacetic acid. The residue was then digested with 8 ml. of 30% KOH and the resulting solution analysed. A larger quantity of dried yeast was used for the examination of the phosphate compounds within the preparation. The dried-yeast suspension (35 ml.; 100 mg./ml.) was spun down after treatment with ^{32}P, and quickly washed with water. The residue was extracted three times with 10 ml. of 10% trichloroacetic acid at 5°. The barium-insoluble, ethanol-insoluble phosphates (Umbreit *et al.* 1949) were chromatographed after treatment with Dowex 50 in the sodium form. About 40μl. of the resulting solution (usually 8–12 ml.) was applied to the chromatogram.

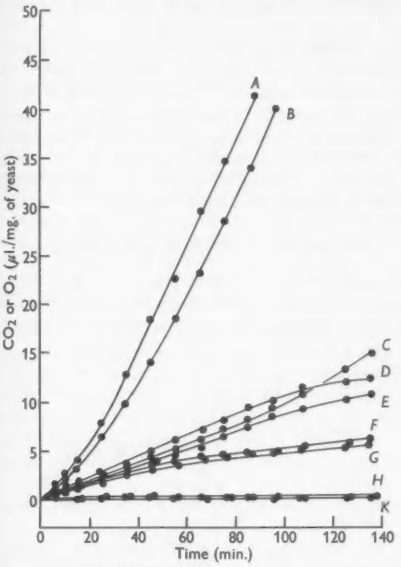

Fig. 1. Gas exchanges of washed dried yeast with no substrate, with glucose and with hexose diphosphate. Each Warburg flask contained 5 mg. of dried yeast (equivalent to 24 mg. of live yeast) in 2 ml. of buffer, pH 5·5. A volume (0·5 ml.) of this buffer, containing substrate, was tipped from the side arm at zero time to give a final substrate concentration of 0·1 M. In anaerobic experiments, the flasks were flushed with N_2; in aerobic experiments, with air. Temperature, 25°. *A*, Glucose anaerobic CO_2; *B*, glucose aerobic CO_2; *C*, glucose O_2; *D*, hexose diphosphate O_2; *E*, hexose diphosphate aerobic CO_2; *F*, endogenous O_2; *G*, endogenous aerobic CO_2; *H*, hexose diphosphate anaerobic CO_2; *K*, endogenous anaerobic CO_2.

RESULTS

Respiration and fermentation of dried yeast

The gas exchanges of the dried yeast with or without substrate (glucose or hexose diphosphate) are shown in Fig. 1. With no substrate no anaerobic production of CO_2 could be measured, whereas in air a definite rate of respiration was observed, with an R.Q. of 1·0 (equal O_2 consumption and CO_2 production). With hexose diphosphate as a substrate, a similar result was obtained. Anaerobic production of CO_2 was essentially zero, whereas respiration proceeded at a rate considerably above endogenous. Again the rates of consumption of O_2 and of production of CO_2 were similar. With glucose as a substrate the pattern was quite different. The rate of respiration of glucose was similar to that of hexose diphosphate but, in contrast, the rates of anaerobic and of aerobic CO_2 production were very high, about five times that of O_2 consumption.

The rate of anaerobic CO_2 production and O_2 consumption by washed dried yeast in the presence of various substrates are given in Table 1. Only in the presence of sugars, glucose, mannose and fructose was there a high rate of anaerobic CO_2

Table 1. *Anaerobic production of carbon dioxide and consumption of oxygen by washed dried yeast in the presence of various substrates*

Results represent averages of three to five determinations. Flasks contained 20 mg. of dried yeast in 2 ml. of buffer, pH 5·5–6·0. A volume (0·5 ml.) of buffer containing substrate was tipped in at zero time to give a final substrate concentration of 0·1 M. Temperature, 25°. In anaerobic experiments the flasks were flushed with N_2, in aerobic experiments with air.

Substrate	Anaerobic production of CO_2 (μl./mg./hr.)	Consumption of O_2 (μl./mg./hr.)
None	0·2	2·4
Glucose	46·0	8·2
Mannose	37·0	6·2
Fructose	40·0	6·4
Galactose	0·2	8·1
Hexose diphosphate	0·2	8·0
Glucose 1-phosphate	0·2	4·8
Glucose 6-phosphate	0·2	3·5
Glycerophosphate	0·2	2·2
Pyruvate	0·2	10·0

production, whereas in the presence of galactose, sugar phosphates, pyruvate and endogenous stores, although the rate of respiration was about the same as that for the sugars, there was only a very low rate of anaerobic CO_2 production.

The glucose disappearance from 0·01 M-solution in the presence of 10 mg. dry wt. of dried yeast/ml. in air or in N_2 was about 90 % in 2 hr. The gas exchanges could account for 80 % of the glucose removed in air and 70 % in N_2. Thus most of the glucose which disappears can be accounted for as CO_2 production or O_2 consumption. The end products of glucose breakdown by the dried-yeast preparation were primarily CO_2 and ethanol. The ethanol was not only measured analytically by the chromate method (Westerfield *et al.* 1942) but was identified by the formation of a derivative and the determination of mixed melting points. On the other hand, little glycogen was formed.

Even though hexose diphosphate, at the same concentration as glucose, gave little or no anaerobic CO_2, it disappeared to the extent of 50 % in 2 hr. The hexose diphosphate that is removed does not appear as pyruvate. In fact, when the dried yeast is suspended with 0·01 M-pyruvate, 70 % disappears within 2 hr. Both substrates disappear at the same rate in air and in N_2, and the loss is not nearly accounted for by the quantity of O_2 consumed.

Effect of washing on metabolism

The experiments of Table 1 were carried out with the dried-yeast residue remaining after washing once with 100 vol. of water. If the yeast was not washed, the pattern of metabolism was the same, with one exception. The unwashed yeast possessed the capacity to produce CO_2 from pyruvate under anaerobic conditions owing to the presence of a soluble carboxylase, as reported previously (Barron, Ardao & Hearon, 1950). However, the rates of metabolism of the washed residues were only half of those before washing. The reduced rates are attributable in large part to the loss of electrolytes, particularly K^+ ions, shown to be necessary by Meyerhof & Kaplan (1951). The K^+ ion-dependence could also be demonstrated by dialysis, or by treating the suspension of dried yeast with a Na^+ cation-exchange resin. The depressed rates with washing, dialysis or resin-treatment could be largely but not completely restored by the addition of 0·1 M-K^+ ions. Mg^{2+} ions also had a slight stimulating action in restoring the rate of fermentation of the washed yeast. Other additions had no pronounced effect on the rate of fermentation. These included adenosine triphosphate (ATP), adenylic acid, diphosphopyridine nucleotide (DPN), boiled-yeast juice, Ca^{2+} ions, Na^+ ions and inorganic phosphate. In this respect the slowly dried yeast differs from the quickly dried yeast of Meyerhof & Kaplan (1951), the fermentation of which is increased by ATP, DPN and boiled-yeast juice.

The dependence of the dried yeast on K^+ ions is also related to its dependence on pH, as shown in Fig. 2. For comparative purposes, data on live yeast are also included (see also Rothstein & Demis, 1953). Fermentation by the dried yeast was less tolerant of low pH than that by live yeast. Furthermore, the fermentation by dried yeast was stimulated by K^+ ions in the range pH 3·5–6·0, whereas that by live yeast was stimulated at values below pH 3·5. The rate of fermentation by the dried yeast was somewhat lower than that of live yeast. For example, in Fig. 2, the ratio of rates (dried to live) was 0·35 without K^+ ions and 0·55 with K^+ ions (at pH 5·0). The respiration of glucose was reduced to a much greater degree than was the fermentation. The maximal ratio of rates (dried to live) was only 0·15.

The ability of the fermentation of dried yeast to proceed rapidly is associated with the integrity of an insoluble structure. Treatment with a homogenizer, blendor or ultrasonic oscillator solubilizes the enzymes, but results in a marked depression in the rate of fermentation as well as a marked change in the pattern of metabolism. For example, hexose diphosphate can be fermented.

Permeability of dried yeast to phosphates

The dehydration–rehydration process used in the preparation of the dried yeast alters the cells in a variety of ways. They can no longer divide. Not

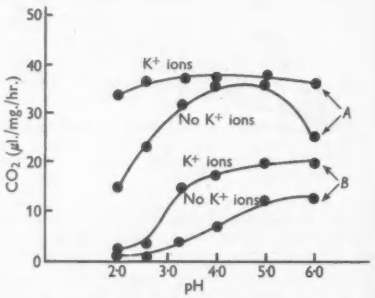

Fig. 2. Effect of potassium on the fermentation of dried and live yeast at different pH values. Each Warburg flask contained 5 mg. of dried yeast (equivalent to 24 mg. of live yeast) or 10 mg. of live yeast in 2 ml. of buffer at the appropriate pH. A volume (0·5 ml.) of this buffer, containing glucose and K^+ ions, was tipped from the side arm at zero time to give a final concentration of 0·1 M-glucose and 0·1 M-K^+ ions. The flasks were flushed with N_2. Temperature, 25°. *A*, Live yeast; *B*, dried yeast.

only is the pattern of metabolism and the dependence on extracellular electrolytes altered but drastic changes in permeability were also observed. Washing with water (4 × 100 ml./g. of dried yeast) removed as much as 25 % of the dry weight, much of it accounted for by the soluble constituents of the cytoplasm such as potassium, phosphates, soluble proteins, organic acids and amino acids. For example, with potassium, 80 % was extracted, reducing the level from 0·14 mole/kg. of original cell material to 0·3 mole/kg. The release of anions measured in terms of buffer capacity in the range pH 4–8 was about the same as that for K^+ ion, 0·13 mole/kg. A large part of the buffer release was due to phosphate compounds and to organic acids such as succinic and acetic acids. As indicated previously, carboxylase activity was readily washed out of the dried yeast. Additional protein to the extent of about 3 % of the original yeast material (on a wet-weight basis) was also solubilized.

The extraction of phosphate materials was studied in some detail (Table 2). For example, four successive washes of 100 ml./g. of dried yeast removed 20 m-moles of orthophosphate/kg. of original cells, essentially all of the inorganic phosphate of the cell. Additional extractions with trichloroacetic acid removed almost no additional phosphate. However, the orthophosphate washed out rather slowly. The duration of the washing period in this experiment was 30 min., and a ratio of wash to solid of 100 to 1, yet the first wash contained only 50–60 % of the phosphate. Nor was the slow release due to a slow liberation of phosphate from organic esters by phosphatases, because phosphate was released at almost the same rate at 0° and 25° (Table 2). Furthermore, no phosphatase activity could be detected by adding phosphate esters to the preparation. Presumably, the outward diffusion of phosphate was impeded by the presence of cellular structures, perhaps precipitated by the dehydration procedure.

During the fermentation of glucose the behaviour of the dried yeast toward phosphates was quite different from that in the absence of glucose (Fig. 3). The release of phosphate esters was blocked and the inorganic phosphate was rapidly absorbed rather than released. If ^{32}P-labelled orthophosphate was used in the experiment, no changes in the specific activity of the phosphate in the supernatant occurred, indicating that there was no phosphate exchange between the medium and the residue. Furthermore, the absorbed phosphate was found to be in the form of phosphate esters rather than inorganic phosphate, as shown by analysis of a trichloroacetic acid extract of the dried yeast. By the use of ^{32}P-labelled orthophosphate and two-dimensional paper chromatography, many of the trichloroacetic acid-extractable esters formed during the process of phosphate absorption were identified. Included were ATP, the sugar phosphates (glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate and especially hexose diphosphate), as well as a variety of incompletely identified substances, mainly nucleotides and triose phosphates (identification based on published R_F values [Burrows *et al.* 1952; Wade & Morgan, 1955]).

The amounts of phosphate esters formed are small compared with the amount of glucose consumed. For example, in Fig. 3, the total phosphate absorbed in 2 hr. was 10 m-moles/kg. of original live yeast. The amount of glucose absorbed in the same period was 600 m-moles/kg. Thus the phosphate uptake is less than 2 % of the sugar uptake. The phosphate esters already present in the dried-yeast residue, and those newly formed by esterification of absorbed phosphate, must be capable of

Table 2. *Cumulative release of inorganic phosphate by dried yeast with washing*

Volume of washing solution was 100 ml. for 1 g. of dry yeast. Water was used for washes 1–4 and trichloroacetic acid (10 %) for washes 5 and 6. Washing time was 30 min. All values are in m-moles/kg. of yeast, based on original wet weight, and are the sum of amounts in the wash together with that in each preceding wash.

	Temperature	
Wash	0°	25°
1	10·4	11·9
2	14·0	16·9
3	17·0	19·0
4	18·4	19·5
5	19·8	20·8
6	20·0	21·0

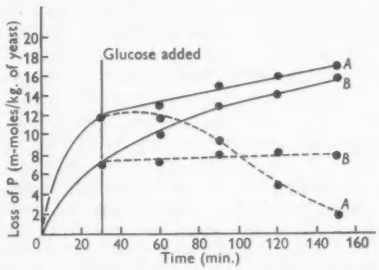

Fig. 3. Gain or loss of inorganic or ester phosphate by dried yeast with or without glucose. The dried-yeast concentration was 16·7 mg./ml. (equivalent to 80 mg. of live yeast/ml.); 0·05 M-glucose when added, and 0·02 M-triethylamine–tartaric acid–succinic acid buffer, pH 5·0. Temperature, 25°. *A*, Orthophosphate; *B*, ester phosphate. Broken line, glucose in the medium; continuous line, glucose-free medium.

turning over rapidly during the fermentation of glucose, accounting for the lack of dependence on the orthophosphate concentration of the medium.

All of the ^{32}P-labelled ester phosphates formed during the absorption of orthophosphate were trapped within the yeast residue, none being detectable in the medium during the course of the experiment. The internal labelled esters did not exchange with unlabelled phosphate esters added to the medium. Thus the fermenting structure is impermeable to phosphate esters, a property which accounts for its inability to ferment sugar phosphates, even though it can utilize glucose, producing alcohol and CO_2, with sugar phosphates as intermediates.

A distribution study with glucose 1-phosphate and glucose 6-phosphate indicated that 87 % of the original cell volume is freely permeable to these substances (compared to 11 % for live yeast). Thus the sugar phosphate-impermeable space, in which the fermentative enzymes are located, must occupy 13 % or less of the original cell. This value is, of course, based on the assumption that the volume occupied by the various cellular structures after rehydration is equal to the original volume.

DISCUSSION

Although in the dried yeast the integrity of the cell as a whole is destroyed so that cell division cannot occur, and soluble constituents of the cytoplasm leak out, the insoluble residues retain many of the properties of the intact cell in so far as fermentation is concerned. In both the live and dried yeast the sugars, glucose, fructose and mannose are fermented rapidly; but other substrates such as endogenous stores (Stier & Stannard, 1936; Spiegelman & Nozawa, 1945), galactose—unless cells are adapted (Spiegelman, Reiner & Cohnberg, 1947), sugar phosphates—unless first split by cellular phosphatases (Rothstein & Meier, 1949), and pyruvate (Barron *et al.* 1950) do not produce carbon dioxide under anaerobic conditions.

Another similarity between the dried-yeast residues and live yeast is the behaviour toward inorganic phosphate and phosphate esters. Both absorb, inorganic phosphate without exchange (Goodman & Rothstein, 1957), with the formation of ester phosphates. Both the fermenting residue of dried yeast and the live yeast cell are impermeable to inflow or outflow of sugar phosphates (Rothstein & Meier, 1949).

The enzymes responsible for fermentation are readily solubilized by homogenization, blending, grinding and similar procedures. They remain in an insoluble residue in the dried-yeast preparation either because they were originally present in the cells as a structural element which has been preserved, or because they have been precipitated as an artifact in the course of the dehydration process. The latter possibility seems unlikely on several grounds. First, dehydration at room temperature is a relatively mild treatment. In Nature, it is not uncommon for certain cells such as those of the Protozoa, spores, slime moulds and seeds to withstand drying, which emphasizes that certain structures can withstand drying without loss of function and that the treatment need not be drastic. Presumably, little chemical alteration is involved, although physical alteration and changes in solubility may certainly occur. These physical changes may be the major factor responsible for any differences between the properties of the intact cell and the preparation produced by drying. Nevertheless, the treatment should be mild enough to allow part of the organization of the cell to remain intact. Secondly, it seems highly unlikely that a series of at least nine soluble enzymes, cofactors and intermediates should be precipitated together in a highly organized system capable of carrying out fermentation at rates approaching that of the intact cell, with no dependence on added orthophosphate, with a rapid turnover of the phosphorylated intermediates and an impermeability to phosphate esters and to pyruvate. Thirdly, Lampen* (personal communication) has found that, with certain yeast samples but not with others, broken-cell preparations obtained by grinding the cells with glass beads showed all the glycolytic activity in the particulate fraction. On more extensive grinding of the preparations, or by the use of higher temperatures or partial autolysis, completely soluble extracts were obtained.

There is little evidence of the exact nature of the structural entity. The glycolytic enzymes could be present in solution within a permeability barrier or within a gel-like structure. The fact that it is unaffected by the dehydration and by treatment with acetone suggests that the idea of a membrane enclosing a solution is less probable than some kind of matrix.

Many recent studies indicate that respiratory activities of cells are associated with mitochondria. In the present studies certain differences between respiration and fermentation suggest that the fermentative structure is distinct from the mitochondria. The dried-yeast preparation will respire glucose, but at a much lower rate, relatively speaking, than it will ferment. Also, the Pasteur effect is weak and there is a high rate of aerobic fermentation. Several substrates, such as hexose diphosphate and pyruvate, that have only a very low rate of production of carbon dioxide under anaerobic conditions, are respired at the same rate

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as glucose. Yet neither of these can enter the fermentative structure. The respiration must proceed in a location different from that of the fermentation. The metabolic pathway involved in the respiratory activity of the dried yeast is of some interest. If the glucose or hexose diphosphate is converted into pyruvate via the Embden–Meyerhof scheme, followed by pyruvate oxidation via the Krebs cycle, then it must be postulated that there are two distinct locations of the glycolytic enzymes in the cell. On the other hand, the respiration may proceed via the phosphogluconate shunt and the pentose cycle. No choice between the two alternatives can be made at present. The rate of respiration of pyruvate is as fast as that of glucose and hexose diphosphate, so the Embden–Meyerhof pathway cannot be excluded. On the other hand, the shunt pathway is known to account for 13–30% of the respiratory activity of the live yeast cell (Beevers & Gibbs, 1954; Blumenthal, Lewis & Weinhouse, 1954; Wang, Gregg, Forbusch, Christenson & Cheldelin, 1956) and the rate of respiration of the dried yeast is only 10–15% of that of the original intact yeast. The shunt pathway could therefore account for the respiration.

Although the loss of hexose diphosphate might be accounted for by breakdown through at least part of the Embden–Meyerhof pathway, there is no evidence that the full course of fermentation is completed. The breakdown of hexose diphosphate, by unwashed dried yeast (where carboxylase activity is present) remains unaltered in rate under aerobic or anaerobic conditions and is neither associated with an anaerobic production of carbon dioxide nor is it associated with an accumulation of pyruvate. In fact, pyruvate is rapidly removed under anaerobic conditions with little production of carbon dioxide. Although this is not direct evidence, it does seem likely that enzymes associated with the insoluble residue are those responsible for at least the major part, if not all, of the fermentation observed in the intact cell.

The fermentative activity of the living yeast cell is apparently located in the periphery of the cell. The evidence is based on a variety of data which are reviewed in detail elsewhere (Rothstein, 1954*a*, *b*, 1955). Briefly, the fermentative activities are directly influenced by extracellular concentrations of H^+ and K^+ ions, rather than by intracellular concentrations of the same ions. Not only are the rates of fermentation affected, but the end products as well. Other evidence is concerned with the close coupling of fermentative reactions with the absorption of phosphate. In cells other than yeast, evidence of a different nature has been cited for the peripheral location of glycolytic activity. Of immediate interest is a recent paper of Shaw & Stadie (1957) on muscle, which indicates two locations for sugar metabolism: a peripheral one concerned with glycolysis and an internal one concerned with other pathways. One presumes that the structural entity described above is also peripherally located in the intact yeast cell.

SUMMARY

1. Baker's yeast was slowly dried, lyophilized and extracted with acetone. The yeast thus treated was unable to divide, and many of its permeability barriers were largely destroyed so that a large proportion of the soluble constituents of the cytoplasm could be washed out.

2. After washing, a residue remained which could ferment glucose to carbon dioxide and ethanol at a rate about half of that of the intact cell. Mannose and fructose could also be fermented, but sugar phosphates, triose phosphates, pyruvate and endogenous stores of carbohydrate produced no carbon dioxide under anaerobic conditions. Respiration of glucose proceeded at a much lower rate. Hexose diphosphate, galactose, pyruvate and endogenous carbohydrates were also respired.

3. The metabolism of the dried-yeast preparations was dependent on electrolytes, particularly K^+ and H^+ ions.

4. The dried yeast, during fermentation of glucose, absorbed inorganic phosphate without exchange, forming the expected phosphorylated intermediates of the glycolysis system. However, the latter, once formed, could not escape from the cell residues.

5. From the properties of the dried-yeast residues, it was concluded that the enzymes for fermentation exist in the cell within an organized structure distinct from the mitochondria.

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Respiratory Enzyme Studies in *Tetrahymena pyriformis*

4. STABILIZATION OF ELECTRON-TRANSPORT COMPONENTS*

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Early attempts to demonstrate an active succinoxidase system in cell-free homogenates of the ciliated protozoan *Tetrahymena pyriformis* were unsuccessful when preparations were made in a glass homogenizer or Waring Blendor with the use of sea sand, Ballotini, alumina or different grades of quartz. Such homogenates were either completely inactive or consumed very little extra oxygen in the presence of added succinate. However, the passage of whole cells through a Logeman homogenizer yielded preparations with quite active electron-transport systems as reflected by succinoxidase, succinic cytochrome *c* reductase, reduced diphosphopyridine nucleotide oxidase, reduced diphosphopyridine nucleotide cytochrome *c* reductase and diaphorase activities (Eichel, 1954, 1956*a*, *c*). On the other hand, kinetic studies of enzymes in these homogenates are seriously handicapped by the extraordinary instability of the electron-transport systems present in the particles, and purification of the enzymes noted above is rendered exceedingly difficult. Although the marked lability of the electron-transport chains argued for seeking other methods of cell disruption which might give more stable and perhaps standardized enzyme preparations, it was felt that the reasonably high initial activities of these homogenates, and the great ease with which they could be prepared without the use of 'contaminating' grinding materials, spoke for a search for means of stabilizing them to facilitate certain types of enzymic studies. In addition, an effective stabilizing agent or group of agents might be expected to yield information about the nature of instability of the particulate electron-transport systems. In part 3 of this series (Eichel, 1956*b*), as part of a study of some properties of the reduced diphosphopyridine nucleotide oxidase system, it was reported that the latter was stabilized for brief periods by suspending and homogenizing whole cells in alkaline phosphate buffer. This procedure, however, resulted in a 50–60 % loss of the initial activity.

This paper deals with the use of two types of agents which have been found to be effective stabilizers of the entire electron-transport systems encompassing the span of either succinate or reduced diphosphopyridine nucleotide to oxygen, as well as of glutamic and β-hydroxybutyric dehydrogenases. One is a relatively thermostable diffusate obtained from commercial preparations of

* Part 3: Eichel (1956*b*).

crude bovine albumin and from whole blood serum, proteose peptone and yeast extract; the other is a group of at least three compounds of which succinic acid is most effective and *iso*citric and citric acids are somewhat less so.

EXPERIMENTAL

Cultures and preparation of homogenates. Experiments were carried out with cells of the S strain of *Tetrahymena pyriformis*, obtained as previously described (Eichel, 1954). Homogenates were prepared by passing freshly harvested cells through a stainless-steel Logeman (C. W. Logeman Company, New York, U.S.A.) homogenizer twice at 5°. This is a hand-operated apparatus in which the cells are broken by being forced through a restricted orifice during the application of pressure on a piston.

Enzyme assays. In the standard succinoxidase and succinic dehydrogenase assays, oxygen consumption was determined at 30° in air by the conventional Warburg manometric method. Each vessel contained in the main compartment, in a final volume of 2·8 ml., sodium succinate, 0·054 M; K_2HPO_4–KH_2PO_4 buffer, 0·036 M, pH 7·1; $AlCl_3$–$CaCl_2$, 0·32 mM; and *Tetrahymena* homogenate, with or without various compounds, 0·5 ml. The centre well contained 0·2 ml. of 20 % (w/v) KOH and filter paper. Al^{3+} and Ca^{2+} ions were added because it had been shown previously that with them the activity of the succinate-oxidizing system was maintained at a somewhat higher level (+16 %) over a period of 1 hr. (Eichel, 1954). In the succinic dehydrogenase assay, each vessel contained, in addition to the above components, 2 mM-KCN and either 0·5 mg. of phenazine methosulphate or 4 mg. of methylene blue chloride. In the rat-liver succinoxidase assay, the conditions were identical except that each vessel also contained 0·5 mg. of cytochrome *c* and 0·4 ml. of a 1:24 water homogenate of liver in place of the protozoan preparation. For the manometric assays of glutamic oxidase and β-hydroxybutyric oxidase, each vessel contained in the main compartment, in a final volume of 2·8 ml., either L-glutamic acid or sodium β-hydroxybutyrate, 0·054 M; 2 mg. of oxidized diphosphopyridine nucleotide (DPN); K_2HPO_4–KH_2PO_4 buffer, 0·036 M, pH 7·1; and *Tetrahymena* homogenate, 0·5 ml. In manometric assays for glutamic and β-hydroxybutyric dehydrogenases, each vessel also contained 2 mM-KCN and 0·5 mg. of phenazine methosulphate. The vessels were immersed in chopped ice during the pipetting of the reaction mixtures and were usually allowed to equilibrate for 10 min. (5 min. with shaking) before closing the manometers. Readings were taken after 5 and 10 min. and at intervals of 10 min. thereafter for 1 hr.

For the standard reduced diphosphopyridine nucleotide (DPNH) oxidase assay, a model DU Beckman spectrophotometer, equipped with thermospacers, was employed at a temperature of 25°. To each 1 cm. cuvette, in the order given, were added the following components which, with the exception of homogenate, had been pre-incubated at 25°: 0·025 or 0·05 ml. of *Tetrahymena* homogenate containing an average of 1·65 mg. of N/ml.; K_2HPO_4–KH_2PO_4 buffer, 0·033 M, pH 8·2; water to a final volume of 3 ml.; DPNH, 0·056 mM (0·12 mg.). Readings (340 mμ) were taken at intervals of 15, 30 or 60 sec. for at least 2–3 min. Because of the marked loss of activity of control homogenates with time (Eichel, 1956*b*) it was necessary to run frequent checks of these samples simultaneously with the treated or fortified homogenates. The DPNH cytochrome *c* reductase assay was carried out like the DPNH oxidase test except that each cuvette also received 1 mg. of pure cytochrome *c*, and readings were taken at 15 sec. intervals for 1 min. at 550 mμ. The addition of cyanide to block the reoxidation of reduced cytochrome *c* is not required (Eichel, 1956*a*). Glutamic and β-hydroxybutyric dehydrogenases were also assayed spectrophotometrically by following DPN reduction at 340 mμ in the presence of cyanide to block the DPNH oxidase system (Eichel, 1956*a*). Each cuvette contained either glutamic acid or β-hydroxybutyrate, 17 mM; KCN, 2 mM; K_2HPO_4–KH_2PO_4 buffer, 0·033 M, pH 7·1; water to 3 ml.; and 1 mg. of DPN. For the glutamic dehydrogenase reaction, in which the rate decreased rapidly with time, six or seven readings were made within about 1 min. Similar readings were taken for the β-hydroxybutyric dehydrogenase reaction, which was linear over a considerable period and more than three times as fast as the reduction of DPN by glutamate. Although both dehydrogenases were assayed at pH 7·1, this does not necessarily represent the optimum pH for these enzymes, i.e. β-hydroxybutyric dehydrogenase is more active at pH 8·2 than 7·1. Fumarase was assayed spectrophotometrically at 25° by the method of Racker (1950). The substrate was 0·033 M-malic acid and the pH was 7·1. Nitrogen determinations were carried out in duplicate by a micro-Kjeldahl procedure.

Materials. The DPNH used in these studies was prepared enzymically by the Sigma Chemical Co., St Louis, Mo., U.S.A. The DPN and cytochrome *c* were also products of the Sigma Chemical Co. The crude albumin preparation was obtained from the Amend Drug and Chemical Co., New York, U.S.A.

RESULTS

Effect of ageing homogenates at 0° on the activities of succinoxidase and succinic dehydrogenase

The succinoxidase data presented in Table 1 were typical of very many assays made with homogenates prepared from cultures of varying ages. Since oxygen uptake was linear for only 10–20 min. and fell off rapidly thereafter, the first 10 min. reading was taken as the most reliable index of activity. The 60 min. readings are given also to indicate the decline in rate. When homogenates were stored at 0°, they lost 30–60 % of the original activity in 1 hr. By 5 hr. 75–90 % of the activity disappeared and after a day all activity was gone. However, when succinic dehydrogenase was measured with either methylene blue or phenazine methosulphate as the electron acceptor, there was only slight inactivation after 5 hr. After 24 hr. the two dye systems responded somewhat differently; 60–90 % of the activity towards methylene blue was lost but only 50–60 % of that towards phenazine methosulphate disappeared. The greater stability of the phenazine system as compared with the methylene-blue system is a second indication that the methylene-blue system includes at least one

Table 1. *Effect of ageing* Tetrahymena pyriformis *homogenates at 0° on succinoxidase and succinic dehydrogenase activities*

Enzyme activities were measured by the standard assays described in the Experimental section. Oxygen uptake values are given for the first 10 min. after closing the manometers and for the first hour. The homogenate added to each vessel contained 1·04 mg. of N. SO, Succinoxidase; SDH, succinic dehydrogenase.

Age (hr.)	Enzyme system	Electron acceptor	Uptake of O_2 (μl.) 10 min.	Uptake of O_2 (μl.) 60 min.	Activity lost (%) 10 min.
0	SO	O_2	47	167	—
	SDH	Methylene blue	49	122	—
	SDH	Phenazine methosulphate	53	130	—
1	SO	O_2	19	95	60
	SDH	Methylene blue	45	131	8
3	SO	O_2	12	50	75
	SDH	Methylene blue	42	143	14
5	SO	O_2	9	42	80
	SDH	Methylene blue	42	132	14
24	SO	O_2	0	5	100
	SDH	Methylene blue	5	26	90
	SDH	Phenazine methosulphate	23	84	57
48	SDH	Methylene blue	0	9	100
	SDH	Phenazine methosulphate	21	80	60

more component than the phenazine system. Previously it had been shown that whereas Atabrine greatly inhibited succinoxidase activity and partially inhibited succinic dehydrogenase with either methylene blue or brilliant cresyl blue, it had no effect on the dehydrogenase as measured by the phenazine system (Eichel, 1956*c*). The differential behaviour of the two carriers towards the particulate dehydrogenase is reminiscent of the findings of Singer, Kearney & Massey (1956) with soluble ox-heart succinic dehydrogenase. They concluded that whereas methylene blue requires at least one additional protein component, phenazine methosulphate reacts directly with the primary dehydrogenase.

It should be emphasized that the stability of the succinate system under study is increased as the complexity of the system is decreased by the use of autoxidizable dyes as terminal carriers in place of oxygen, and this parallels findings made with various DPNH-oxidizing enzymes (Eichel, 1956*b*). Diaphorase (dichlorophenolindophenol acceptor) is more stable than DPNH cytochrome *c* reductase, which, in turn, is considerably more stable than DPNH oxidase (oxygen acceptor); identical fall-off rates are seen with both DPNH oxidase and succinoxidase.

Effect of ageing homogenates at 0° on the activities of glutamic and β-hydroxybutyric oxidases and glutamic and β-hydroxybutyric dehydrogenases

As expected, in homogenates aged at 0° and assayed at varying times in the presence of DPN and glutamate or β-hydroxybutyrate, oxygen consumption declined rapidly and at a rate essentially parallel to the loss of succinoxidase activity. In the dehydrogenase systems, on the other hand, with phenazine methosulphate as the electron acceptor, the activity of aged homogenates towards glutamate and β-hydroxybutyrate fell by only 10–28% and 20–37% after 1 and 2 days respectively. When glutamic and β-hydroxybutyric dehydrogenases were measured spectrophotometrically by following the reduction of DPN at 340 mμ in the presence of cyanide, neither enzyme lost activity for a period of at least 24 hr.

Effect of heating homogenates on the activities of succinoxidase and succinic dehydrogenase

Complete inactivation of the succinoxidase system occurred on heating the homogenates for 5 min. at 37° (Table 2). As with ageing at 0°, succinic dehydrogenase responded somewhat differently, depending upon the carrier used. Heat-treatment for 5 min. at 45° destroyed almost all of the activity towards methylene blue but only a little more than half of that towards phenazine methosulphate, again suggesting that the system which methylene blue tests is more labile and, presumably, more complex. It has been reported (Eichel, 1956*b*) that heating homogenates for 5 min. at 35° also destroyed 90% of the DPNH oxidase. Thus the extreme lability at 0° of both oxidases was accentuated by heating the *Tetrahymena* preparations briefly at relatively low temperature. Glutamic and β-hydroxybutyric oxidases were also inactivated by heating homogenates for 5–10 min. at 30°. Results pertaining to the albumin experiments in Table 2 are described in the next section.

Table 2. *Effect of heating* Tetrahymena pyriformis *homogenates with and without crude bovine blood albumin on succinoxidase and succinic dehydrogenase activities*

Enzyme activities were measured by the standard assays. Immediately after preparation the homogenates were heated as indicated with or without the crude albumin preparation in the final concentrations listed, and quickly cooled and assayed. The controls were unheated homogenates. Each Warburg vessel contained 1·95 mg. of homogenate N in the succinoxidase assay and 2·27 mg. of homogenate N in the succinic dehydrogenase assays.

Enzyme system	Conditions of heating	Uptake of O_2 (μl./10 min.)	Activity lost (%)
Succinoxidase	Control	30	—
	5 min. at 37°	1	97
	10 min. at 30°	5	83
	5 min. at 37° +3% of albumin	25	17
	10 min. at 37° +3% of albumin	19	37
Succinic dehydrogenase			
Methylene blue	Control	56	—
	5 min. at 40°	28	50
	5 min. at 45°	2	97
	5 min. at 40° +2% of albumin	57	0
Phenazine methosulphate	Control	48	—
	5 min. at 40°	32	33
	5 min. at 45°	20	58
	5 min. at 50°	1	98
	5 min. at 40° +2% of albumin	57	0

Table 3. *Effect of ageing* Tetrahymena pyriformis *homogenates at 0° in the presence of crude bovine blood albumin on succinoxidase and succinic dehydrogenase activities*

Enzyme activities were measured by the standard assays. In *A* and *B*, fresh homogenate was added to a suspension of crude albumin in water to give the final albumin concentrations listed. Portions of these mixtures were then added to the Warburg vessels. All mixtures were assayed at the indicated intervals after preparation and were stored at 0° throughout the experiments. Each vessel contained 1·62 mg. of homogenate N.

A. Succinoxidase

Concn. of albumin (%)	Age of homogenate at assay (hr.) 0	4	24	48
	Uptake of O_2 (μl./10 min.)			
0	21	8	0	0
0·5	25	16	4	0
1	25	22	11	2
2	20	25	18	5

B. Succinic dehydrogenase

Electron acceptor	Concn. of albumin (%)	Age of homogenate at assay (hr.) 0	24	48
		Uptake of O_2 (μl./10 min.)		
Phenazine methosulphate	0	35	17	14
	3	54	40	33
Methylene blue	0	30	8	3
	3	49	34	24

Stabilization of succinoxidase, succinic dehydrogenase and reduced diphosphopyridine nucleotide oxidase activities by a crude bovine albumin preparation

The results of a typical experiment, presented in Table 3*A*, show that the addition of a crude bovine blood-albumin preparation to a freshly prepared homogenate, in a final concentration of 2%, prevented the loss of succinoxidase activity for about 24 hr. in homogenate allowed to age at 0°. (In the experiment illustrated, the concentration of the albumin powder in the vessels was 0·4%.) Stabilization could be extended to at least 43 hr. by adding 5% of albumin to a fresh homogenate. In the absence of added succinate, a 2% albumin–homogenate mixture consumed only 4 μl. of oxygen in 10 min. In the absence of both succinate and homogenate there was no oxygen uptake by the albumin preparation.

Succinic dehydrogenase activity, measured with either phenazine methosulphate or methylene blue, was also stabilized (Table 3*B*). Although it would seem plausible that the apparent activation of the dehydrogenase at zero time by the crude albumin powder was due to the oxidation of various substrates present in the powder, with the dyes serving as carriers, the fact that similar results were obtained with crystalline albumin (see below) might indicate some other effect was responsible for the stimulation. Whether the decline in activity at 24 hr. in the presence of the crude albumin represented the loss of this enhanced activity or a partial

loss of the original succinic dehydrogenase or both was not examined.

Stabilization of both succinoxidase and succinic dehydrogenase activities against heat-inactivation, by heating homogenates at 37° or 40° in the presence of the crude albumin preparation, is shown in Table 2.

The DPNH oxidase system is also stabilized by the albumin preparation. The results of Table 4 indicate that in water homogenates about 90 % of the DPNH oxidase activity was preserved in homogenate–albumin mixtures 24 hr. after storage at 0°, and 50 % remained after 2 days. It is possible that higher concentrations (>7·5 %) of the albumin extract would protect the oxidase to a greater degree for a longer time. In other experiments not tabulated, DPNH cytochrome *c* reductase acitivty of homogenates aged at 0° decreased 30–40 % after 6 hr., 45–60 % after 24 hr. and 90 % after 72 hr. The crude albumin stabilized the DPNH cytochrome *c* reductase activity completely for at least 72–96 hr.

Table 4. *Effect of ageing* Tetrahymena pyriformis *homogenates at 0° in the presence of crude bovine blood albumin on reduced diphosphopyridine nucleotide oxidase activity*

DPNH oxidase activity was measured by the standard assay described in the Experimental section. With the 'water homogenate', cells were washed and homogenized in water and 2 ml. portions of homogenate (1·92 mg. of N/ml.) were added to test tubes containing the indicated amount of a lyophilized extract of the crude albumin (see Results section for its preparation). After thorough mixing, each sample was assayed immediately and at the intervals shown. With the 'phosphate homogenate', cells were washed twice in water, once in 0·1 M-KH_2PO_4–K_2HPO_4 buffer, pH 8·2, and homogenized in 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·2. Portions of homogenate were again added to the lyophilized extract and assayed as before. All homogenate mixtures were stored at 0° throughout the experiments. The values are given as $10^3\Delta E_{340\,m\mu}$/2 min.

Albumin added (mg.)	Age at assay (hr.) 0	5·5	24	48
	Water homogenate			
0	191	18	8	4
10	178	153	20	8
30	196	161	84	16
60	170	194	140	62
90	191	193	151	86
120	177	210	151	94
150	175	209	156	94
	Phosphate homogenate			
0	98	113	73	31
5	122	115	73	33
10	123	117	81	45
20	124	119	90	53
30	122	118	94	59
60	117	125	109	75

It had been pointed out previously that homogenizing whole *Tetrahymena* cells in alkaline phosphate buffer prevented the loss of DPNH oxidase activity for periods up to 24 hr. The results in Table 4 show that the stabilizing action of the alkaline phosphate and of the crude albumin are essentially additive and suggest that stabilization of the oxidase for longer periods might be achieved by combination of the two agents and increasing the albumin concentration.

Stabilization of the succinoxidase system of rat-liver homogenate by the crude bovine-albumin preparation

It was of interest to determine whether the albumin preparation could also stabilize the aerobic oxidation of succinate by a mammalian tissue. Rat-liver homogenate (5 ml.; 1·3 mg. of N/ml.) was mixed with 5 ml. of a 1 % suspension of albumin. A control, without albumin, was similarly prepared. Whereas the rat-liver-homogenate system was far more stable than that of *Tetrahymena*, there was protection against the loss of succinoxidase activity on ageing at 0°. At 48 and 120 hr., when 45 and 91 % of the control activity was lost respectively, only 13 and 42 % of the activity of the albumin-protected homogenate disappeared.

Specificity of the crude bovine-albumin preparation

To determine whether the stabilization conferred by the crude albumin preparation was characteristic of proteins in general, several purified proteins were tested with the *Tetrahymena* succinoxidase system. The results are presented in Table 5. Whereas crude preparations of haemoglobin and human serum albumin had some stabilizing activity, neither purified (fraction V powder) nor crystallized bovine plasma albumin was effective. In fact, at 2 % the latter preparations were inhibitory at zero time. Crystalline albumin was not only incapable of preventing the loss of succinic dehydrogenase activity on ageing at 0°, but after 24 hr. was markedly inhibitory. At zero time, however, the crystalline albumin stimulated succinic dehydrogenase considerably with the phenazine methosulphate and methylene blue tests. Both proteose peptone and yeast extract gave considerable protection of the succinoxidase system. In the presence of 1 or 2 % of peptone or yeast extract, homogenates lost little activity after ageing for 5 hr. at 0°, but lost 50 % or more after 24 hr. The glutamic and β-hydroxybutyric oxidase systems were both stabilized by the yeast extract and the peptone.

From the results of Table 5 it can be concluded that the stabilizing action of the crude albumin preparation (Table 3) is due to an impurity and not to the albumin. The presence of trace amounts of this material is suggested in the haemoglobin and

Table 5. *Effect of ageing* Tetrahymena pyriformis *homogenates at 0° in the presence of various purified proteins on succinoxidase activity*

Succinoxidase activities were measured by the standard assay. Homogenates were added to the proteins to give the final protein concentrations listed. Portions of these mixtures were then added to the Warburg vessels, and the mixtures were reassayed after being stored for 24 hr. at 0°.

Protein	Concn. (%, w/v)	Activity lost after 24 hr. (%)
Gelatin	2, 3, or 4	100
Haemoglobin*	2, 3, or 4	85
Human serum albumin†	0·5, 1, 2, or 3	76 (at 3%)
Human serum γ-globulin‡	0·5, 1, 2, or 3	95 (at 3%)
Proteose peptone§	1 or 2	53
Casein	1 or 2	100
Purified bovine-plasma albumin‖	0·5, 1, 2, or 3	100**
Crystalline bovine albumin¶	0·13, 0·5, 1, 2, or 3	100**

* Nutritional Biochemicals Corp.
† Fraction V; E. R. Squibb and Sons.
‡ Fraction II; E. R. Squibb and Sons.
§ Difco.
‖ Fraction V; Armour and Co.
¶ Four times recrystallized; Armour and Co.
** At the 2 and 3% levels, 30–40% inhibition occurred at zero time.

human serum-albumin samples used; proteose peptone and yeast extract (not tabulated) apparently contain a considerable amount of it.

Presence of the stabilizing activity in blood

Since the supply of the crude albumin preparation, which was at least 2–3 years old, was limited, a newly received lot of the crude bovine albumin was tested and found to contain the stabilizing activity. In the absence of information about the method of preparation of the crude albumin, it was important to determine whether its stabilizing activity was due to a naturally occurring material or one added during the course of separation of the albumin from blood. Hence blood serum and plasma from several sources were tested for stabilizing activity towards the succinoxidase system of aged *Tetrahymena* homogenates. When 2·5 ml. of diluted rat serum (1 ml. of serum plus 1·5 ml. of water) were added to 2·5 ml. of homogenate (2 mg. of N/ml.), 70–100% of the succinoxidase activity was preserved for at least 24 hr. Horse, human and chick serum were all active.

Some properties of the stabilizing material

In the foregoing studies, a known amount of the crude albumin preparation was generally suspended in glass-distilled water and allowed to stand for 1 hr. in the cold before adding the homogenate to it. However, since the albumin preparation was only partially soluble, the following experiment was carried out to determine with which fraction the protective activity was associated. Crude albumin (300 mg.) was suspended in 5 ml. of water, and allowed to stand for 1 hr. in the cold and centrifuged at 110 800 *g* for 20 min. A volume (2·5 ml.) of the pale-yellow supernatant fluid was added to 2·5 ml. of homogenate. The viscous sediment was suspended in 5 ml. of cold water and 2·5 ml. of the suspension added to 2·5 ml. of homogenate. After 24 hr., 50% of the original succinoxidase activity remained in the homogenate–supernatant fluid mixture and 30% in the homogenate–sediment sample, indicating that a considerable amount of the protective material could be extracted by water. On a dry wt. basis, about 15–20% of the crude albumin preparation was extracted under these conditions. The extract could be lyophilized without the loss of its stabilizing activity.

In Table 6 are summarized the results of a number of replicate experiments which show that the stabilizing activity of the albumin extract was unaffected by heating at 100° for 10 min. at either neutrality or pH 4·5, or by adjustment to pH 10·4 briefly. Heating at 100° at pH 10·4, however, appeared to destroy the activity almost completely, although a small amount of protection was achieved in some experiments. Heating albumin extract in more dilute solutions destroyed some of its protective activity, but heating for 30 min. at 100° had no more effect than heat treatment for 10 min. It was also noted that the initial succinoxidase activity of the mixture of homogenate and boiled alkalinized extract was very low, suggesting the possibility that some inhibitory material was released which, in turn, prevented or masked the stabilizing activity. Dialysis of the albumin extract against glass-distilled water resulted in the complete loss of stabilizing activity. Lyophilized diffusate (7 mg./ml. of 1:2 homogenate) was effective in protecting against the loss of succinoxidase activity, although in a few experiments the stabilization was less after 24 hr. than after 5 hr. Heating lyophilized diffusate for as long as 30 min. at 100° caused only a slight decrease in the stabilizing effect. In other experiments not listed in Table 6, it was shown that the stabilizing action of rat serum, yeast extract and proteose peptone

towards succinoxidase and β-hydroxybutyric oxidase was markedly reduced by dialysis, and human blood plasma, heated at 100° for 30 min. and then centrifuged to remove denatured protein, lost none of its stabilizing activity.

Essential irreversibility of the loss of succinoxidase activity

The addition of crude albumin preparation to Warburg vessels containing homogenate which had been aged for 20 hr. at 0° failed to restore the succinoxidase activity, although some oxygen uptake was registered above the control level. This suggested that the loss of succinoxidase activity was largely irreversible and that the activity present at a given time after preparation of the homogenate was a function of the time of addition of the crude albumin preparation. The results presented in Fig. 1 illustrate this effect in greater detail. In this experiment, the water control lost little activity in the first hour, but thereafter activity declined at the more typical rate. Albumin extract added to homogenate at zero time stabilized the succinoxidase activity for at least 5 hr. essentially at the initial level and for 24 hr. at 85% of the initial level. On the other hand, addition of extract to homogenate at 3 or 5 hr. did not restore the activity already lost, but did preserve the activity remaining at the time extract was added. The somewhat higher activities of the extract–homogenate mixtures prepared at 3, 5 and 24 hr. compared with the water control assayed at these times may represent a small reversal of the inactivation.

Table 6. *Effect of different treatments of crude albumin extract on its ability to stabilize the succinoxidase activity of* Tetrahymena pyriformis *homogenates*

Lyophilized albumin extract (700 mg.) was dissolved in 25 ml. of water (pH 7·1). One portion (5 ml.) was brought to pH 10·4 with N-KOH and another to pH 4·55 with N-HCl, and both samples were heated in a boiling-water bath for 10 min. The samples were then chilled and immediately brought to pH 6·5–6·7. A third portion (5 ml.) was adjusted to pH 10·4, allowed to stand for 10 min. at room temperature, and then readjusted to pH 6·6. A fourth sample was simply heated (100°) for 10 min. at pH 7·1, and then chilled, and a fifth was untreated. To 1·5 ml. of each of the above portions of albumin extract was added 1·5 ml. of protozoan homogenate (4·14 mg. of N); each mixture was assayed immediately and after standing for 5 hr. at 0°.

In the experiments dealing with the dialysed extracts and diffusates, 10 ml. of albumin extract (30 mg./ml.) was dialysed against 165 ml. of glass-distilled water in the cold with constant stirring. After 18 hr., 5 ml. of the extract was removed, and the remainder was dialysed for an additional 18 hr. against fresh distilled water. The first diffusate was lyophilized and dissolved in 9 ml. of water. A portion (4 ml.) of this solution was added to 4 ml. of homogenate (2·5 mg. of N/ml.); a portion (2·5 ml.) was heated at 100° for 30 min., and chilled and added to 2·5 ml. of the same homogenate; each mixture and a control were assayed immediately and after standing for 5 hr. at 0°. In this experiment, the lyophilized diffusate was not weighed. Generally, it was found that under these conditions about 42% of the albumin extract was present in the diffusate or, in this case, 126 mg. Therefore 7 mg. of lyophilized diffusate was mixed with each millilitre of diluted homogenate.

Additions to homogenate	Activity lost in 5 hr. (%)
None	85
Extract	0
Boiled extract	0
Alkalinized extract (pH 10·4)	13
Boiled alkalinized extract	70
Boiled acidified extract (pH 4·55)	0
Extract dialysed 18 hr.	86
Extract dialysed 36 hr.	97
Lyophilized diffusate of extract	0
Lyophilized diffusate of extract, boiled 30 min.	17

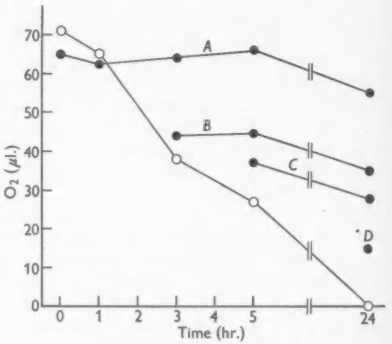

Fig. 1. Effect on succinoxidase activity of adding crude albumin extract to *Tetrahymena* homogenate at varying intervals after preparation of homogenate. Immediately after homogenizing the cells, a portion of homogenate was mixed with an equal volume of water (the control, ○), a second portion was mixed with an equal volume of albumin extract (●), and both samples were assayed immediately for succinoxidase activity and also at 1, 3, 5 and 24 hr. thereafter. Other portions of the original homogenate were also diluted 1:2 with extract at 3, 5 and 24 hr., and each mixture was assayed immediately after its preparation as well as at the indicated times. All homogenates were stored at 0° throughout. Curves *A*, *B* and *C* represent results obtained when the extract was added to homogenate at 0, 3 and 5 hr. respectively. The point labelled *D* represents the activity obtained when the extract was added after 24 hr.

Failure of certain compounds to stabilize the succinoxidase system

Attempts to reproduce the action of the crude albumin preparation by adding to fresh homogenates various compounds, which have been implicated in electron-transport function or which occur in blood plasma, were more or less unsuccessful. Thus adenosine triphosphate (3 mM); cholesterol (4·2 mM); animal or vegetable lecithin (1 mg./ml. of homogenate); riboflavin phosphate (1·7 mM); a mixture of 21 L-amino acids (40 μg. of each in 5 ml. of homogenate); larger concentrations of histidine (32 mM), cysteine (6 mM) or tryptophan (8 mM); uridine triphosphate (0·7 mM); bilirubin (0·1 mg./5 ml. of homogenate); glucose (0·05M, 0·25M); NaCl (0·05M, 0·25M); ascorbic acid (5 mM); Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} ions (all 0·05–5·0 mM); Mn^{2+}, Cu^{+} ions (each 5 mM); and a suspension of haemin (2 mg./ml. of homogenate) failed to stabilize the succinoxidase activity of homogenates aged at 0°. Since the absorption spectrum of the lyophilized diffusate of the crude albumin extract exhibited a very broad plateau in the region 250–280 mμ, a number of nucleic acid derivatives were also tested for activity. Inosine, uridine, cytidine and adenine were without effect, but a mixture of adenylic acid a and b or adenosine alone gave some stabilization and to about the same extent. After 20 hr. at 0°, whereas the control lost 90–96% of the initial activity the adenylic acid- or adenosine-supplemented homogenates lost only 60–80%. Liver-extract powder also partly stabilized the succinoxidase system.

Effect of succinate on the stabilization of succinoxidase and succinic dehydrogenase activities of heated and aged (0°) *homogenates of* Tetrahymena pyriformis

In part 1 of this series (Eichel, 1954), the aerobic oxidation of succinate by homogenates of *Tetrahymena* was studied by equilibrating pre-chilled Warburg vessels, which contained succinate in the side arms and a homogenate–buffer mixture in the main compartments, for 5 min. at 30°, and tipping in the succinate and then closing the manometers after an additional 5 min. Later, it was observed that the oxygen consumption of such a system could be increased considerably during the first 10 min. period simply by adding the succinate to the homogenate and buffer in the main compartments of vessels maintained in an ice bath and then equilibrating the vessels at 30° before closing the manometers. This procedure has been employed in more recent work dealing with the succinoxidase system (Eichel, 1955, 1956*c*). The greater activity obtained by mixing all components at 0° before equilibration at 30° was interpreted as being due either to substrate activation or to substrate protection of the enzyme system, but this point was not pursued further at the time. The demonstration of an extremely rapid inactivation of succinoxidase and DPNH oxidase activities by heating at 30° or by ageing at 0°, and the stabilization of these electron transport systems by a crude blood-albumin preparation or blood serum, prompted a more detailed investigation into the effects of heating and ageing, with and without succinate, on the succinoxidase and DPNH oxidase systems. Homogenate–phosphate buffer mixtures which were equilibrated in the Warburg bath for as little as 6 min. at 30° before receiving sodium succinate lost half of their succinoxidase activity. The degree of inactivation under these conditions was quite variable, but always severe, and generally ranged from 35 to 60% after 5–6 min. However, when 2 ml. of fresh homogenate (4·16 mg. of N) was heated for 10 min. at 30° with succinate in a final concentration of 34·8 mM, and cooled and then assayed under the usual test conditions, there was no loss of enzyme activity compared with unheated homogenate. Heating for 30 min. at 30° with the same amount of succinate caused only a 10% drop in activity, which was unaltered by doubling the succinate concentration. With 67 mM-succinate in the presence of 4·28 mg. of homogenate N, heating for 10 min. at 35° resulted in very little enzyme inactivation (−8%), but at 40° a 59% decrease in activity was found. The latter value was halved when the succinate concentration was raised to 0·231M.

Succinic dehydrogenase activity measured with phenazine methosulphate was also protected when homogenates were heated in the presence of succinate. Under the latter conditions, even after 10 min. at 45°, 44% of the dehydrogenase activity remained; only 17% of the activity was left after heating for 10 min. without succinate.

Addition of succinate to homogenates aged at 0° also prevented the rapid inactivation of the succinoxidase system. Incubation of homogenate (2·05 mg. of N/ml.) with added succinate in a final concentration of 9·16 mM gave complete stabilization for 4·5 hr. and resulted in the loss of only 18% of the original activity after 24 hr. (Table 7). At 0·154 mM-succinate, half of the activity was preserved for 4·5 hr. but none remained after 24 hr. In some cases, complete stabilization was achieved for 48 hr. at 35 mM-succinate. Solutions of succinic acid adjusted to pH 7·0 with either dilute KOH or NaOH behaved like commercial sodium succinate.

After succinate-stabilized homogenates (18·9 mM) were centrifuged at 73 300 *g* for 30 min. and the pellet, containing all of the succinoxidase activity (Eichel, 1955), was resuspended in water, ageing of the glass-homogenized pellet at 0° for 24 hr.

resulted in nearly complete inactivation of the succinoxidase system. However, addition of succinate to the homogenized pellet immediately after its preparation preserved for at least 19 hr. the succinoxidase activity present at the time succinate was added. The abolition of the stabilizing effect by the centrifuging of succinate-supplemented homogenates indicates that the succinate is readily dissociated from its binding site(s) on the particles. In a similar experiment, it was found that when homogenate was stabilized with both succinate (18·9 mM) and lyophilized albumin extract, and centrifuged and the resultant pellet homogenized in water, only about 23 % of the initial activity was present in the pellet after 24 hr. of ageing at 0°.

Table 7. *Effect of ageing* Tetrahymena pyriformis *homogenate at 0° in the presence of sodium succinate on succinoxidase activity*

Portions (4 ml.) of fresh homogenate (2·05 mg. of N/ml.) were incubated with 0·5 M-sodium succinate to give the final concentrations listed. Samples of these mixtures were then added to the vessels. All mixtures were assayed at the indicated intervals after preparation and were stored at 0° between assays. The figures represent uptake of oxygen for the first 10 min. after closing the manometers (*a*) and for the first hour (*b*).

	Age of homogenate at assay (hr.)					
	0		4·5		24	
Final concn. of succinate (mM)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
	Uptake of O_2 (μl.)					
0	35	179	12	71	0	9
17·4	34	174	33	173	33	157
9·16	34	175	33	176	28	117
1·25	35	181	29	148	2	14
0·308	36	180	21	117	0	10
0·154	37	192	17	89	0	11

Effects of other compounds on the stabilization of succinoxidase activity

A number of compounds, many of which are closely related to succinic acid structurally, were tested for their ability to stabilize the complete succinoxidase system in homogenates aged at 0°. They were fumarate, malate, α-oxoglutarate, pyruvate, acetate, malonate, *iso*citrate, citrate, formate, oxalate, tartrate, glutarate, adipate, butyrate, glutamate, succinylcholine chloride, choline, succinimide, mercaptosuccinate and pyrophosphate. None of these compounds caused significant oxygen uptake under the experimental conditions in the absence of added succinate. Of the 20 compounds, only *iso*citrate, citrate, succinylcholine and succinimide gave appreciable protection (Table 8). *iso*Citrate was most effective; at low concentration (35 mM), homogenates lost only 10 % of the enzyme activity after 5 hr. At 0·136 M, *iso*citrate-fortified homogenates lost 42 % of the activity after 24 hr. Citrate was somewhat less effective. Succinylcholine gave almost complete stabilization for 5 hr. at 67 mM, but had little effect at twice this level after 24 hr. Choline alone was ineffective, and did not inhibit the stabilization by succinate. Hence the failure of succinylcholine to protect at 24 hr. could not be due to the presence of any free choline that might have been formed by hydrolysis. Partial protection was conferred by succinimide at high concentration

Table 8. *Effect of ageing* Tetrahymena pyriformis *homogenates in the presence of various compounds on succinoxidase activity*

Results listed are taken from a number of experiments in which many different homogenate preparations were employed. In each experiment, volumes of 0·5 M-solution of the compound to be tested were added to portions (2 ml.) of homogenate to give the final concentrations indicated, and the mixtures were assayed immediately and after 5 hr. (*a*) or 24 hr. (*b*) or both. All of the compounds tested were adjusted to pH 7·0 with either dilute acid or base before adding to homogenate, and all homogenate mixtures were aged at 0° throughout. In each experiment one or more controls, to which several volumes of water were added, were run. Control homogenates lost 65–85 % of their original succinoxidase activity after 5 hr. and 95–100 % after 24 hr. Homogenates aged in the presence of 17·4 mM-succinate lost no activity after 24 hr. Total N content of the homogenates used ranged from 1·60 to 2·20 mg./ml.

	Concn. (mM)							
	18		35		67		136	
Compound tested	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
	Succinoxidase activity lost after ageing for 5 and 24 hr. at 0° (%)							
Sodium *iso*citrate	—	—	10	80	—	—	31	42
Sodium citrate	35	88	42	65	47	65	67	55
Succinylcholine chloride	70	—	51	—	15	87	4	86
Succinimide	70	—	70	—	60	100	46	92
Sodium pyrophosphate	37	—	50	—	—	—	—	—

Table 9. *Effect of ageing* Tetrahymena pyriformis *homogenates in the presence of various compounds on reduced diphosphopyridine nucleotide oxidase activity*

Results listed are taken from a number of experiments in which many different homogenate preparations were employed. Experimental conditions were exactly as described in Table 4. Total N content of the homogenates used ranged from 1·54 to 1·79 mg./ml.

	Concn. (mM)												
	0			18			35		70		136		
	Ageing time (hr.)			Ageing time (hr.)			Ageing time (hr.)		Ageing time (hr.)		Ageing time (hr.)		
	2	5	24	2	5	24	5	24	24	50	2	5	24
Compound tested	DPNH oxidase activity lost after ageing at 0° for time shown (%)												
None	63	89	96	—	—	—	—	—	—	—	—	—	—
Sodium succinate	—	—	—	0	0	25	0	21	16	53	—	—	—
Sodium citrate	—	—	—	41	58	82	—	—	—	—	31	41	62
Sodium *iso*citrate	—	—	—	33	63	86	—	—	—	—	11	15	44
Malic acid	—	—	—	54	79	97	—	—	—	—	—	—	—
Sodium malonate	—	—	—	—	—	—	—	—	—	—	50	70	97

(0·136 M) for 5 hr., but very little protection was obtained after 24 hr. with this compound. Ethylenediaminetetra-acetic acid (63 mM), which has been used to stabilize various enzymic activities in animal and plant preparations, gave negative results, as did gum arabic (1·7 %) and sucrose (0·05–0·25 M).

Many of the compounds listed above inhibited succinoxidase activity at zero time at the higher concentrations and, in some instances, inhibition obtained at low levels prevented the testing at higher concentrations. *iso*Citrate gave 20 and 50 % inhibition at 0·045 and 0·136 M respectively, whereas citrate inhibited 62 % at the latter concentration. Fumarate, malate, α-oxoglutarate, glutarate, adipate and pyruvate inhibited succinoxidase activity by 20–40 % at 6·7 mM. Formate, oxalate and tartrate gave 30–38 % inhibition at 0·136 M. Malonate, as might be expected, gave 35 and 70 % inhibition at 6·2 and 18 mM respectively. At 6·2 mM-malonate, slight protection was achieved. Mercaptosuccinate inhibited activity by 35 % at 18 mM, and succinylcholine inhibited by 30 % at 0·136 M. Pyrophosphate, reported to have a specific protective effect on succinic dehydrogenase and other enzymes (Morton, 1955), stabilized the succinoxidase system considerably after 5 hr. (Table 8), but also inhibited the initial activity by 40 % at the same concentration (18 mM). At twice this concentration, stabilization by pyrophosphate was lessened, and the inhibition rose to 60 %. It should be noted that the concentrations of all compounds tested as potential stabilizers refer only to the final concentrations of the aged homogenate–compound mixtures. The actual concentration of each compound present in the vessels during assay was less by a factor of 5·6.

Effects of succinate and other compounds on the stabilization of reduced diphosphopyridine nucleotide oxidase and reduced diphosphopyridine nucleotide cytochrome c reductase activities of aged and heated homogenates of Tetrahymena pyriformis

Table 9 summarizes a number of experiments in which the effective stabilizers of the succinoxidase system were tested for their ability to protect the DPNH oxidase system against inactivation on ageing at 0°. It can be seen that succinate (18 mM) prevented the loss of DPNH oxidase activity completely for at least 5 hr., and at 70 mM preserved 84 and 47 % of the activity after 24 and 50 hr. respectively. In low concentration, *iso*citrate and citrate were somewhat effective, and in higher concentration, both tricarboxylic acids gave considerable protection, with *iso*citrate surpassing citrate, as in the succinoxidase experiments. In agreement with their effects on succinoxidase activity, malic acid was ineffective as a DPNH oxidase stabilizer at 18 mM and quite inhibitory at higher concentrations, as was malonate (0·136 M).

DPNH cytochrome *c* reductase activity of homogenates aged at 0° was also stabilized by the presence of succinate (not tabulated).

It had been previously reported (Eichel, 1956*b*) that dithionite-reduced DPN (final concentration 0·528 mM) failed to stabilize the DPNH oxidase system of homogenates aged at 0°. It has now been found that homogenates aged with enzymically reduced DPN (final concentration 0·583 mM) lose essentially no activity after 2–3 hr. and only 55 % after a day.

The addition of succinate (18 mM) to homogenate heated for 10 min. at 30° completely prevented the

Table 10. *Effect of ageing* Tetrahymena pyriformis *homogenates in the presence of sodium succinate on glutamic and β-hydroxybutyric dehydrogenase and fumarase activities*

Enzyme activities were measured spectrophotometrically as described in the Experimental section. Portions (3 ml.) of fresh homogenate were incubated with 0·22 ml. of either water or 0·5 M-sodium succinate, and samples of these mixtures were assayed immediately and at the times indicated. The mixtures were stored at 0° throughout.

Enzyme	Succinate added to homogenate	Ageing time (days) 1	2	3	4	5	7	8	10
		Activity lost after ageing at 0° for no. of days shown (%)							
Glutamic dehydrogenase	–	0	0	37	46	52	—	—	—
	+	0	0	12	26	34	—	—	—
β-Hydroxybutyric dehydrogenase	–	0	33	61	65	65	67	82	—
	+	0	0	0	0	0	0	17	33
Fumarase	–	0	0	0	—	10	—	40	70
	+	0	0	0	—	0	—	0	0

inactivation of DPNH oxidase activity, which amounted to 89 % in homogenate heated without succinate.

Glutamic and β-hydroxybutyric dehydrogenases and fumarase

Glutamic dehydrogenase, measured by DPN reduction, was stable for 2 days and lost only 37 % of the initial activity after 3 days, and β-hydroxybutyric dehydrogenase was slightly less stable.

Ageing of *Tetrahymena* homogenate at 0° with succinate completely prevented the loss of β-hydroxybutyric dehydrogenase activity measured by DPN reduction for as long as 4–7 days (7 days for the preparation illustrated in Table 10). Glutamic dehydrogenase activity (DPN reduction) was also stabilized by succinate, but not quite as effectively.

Fumarase, a particulate enzyme (H. J. Eichel, unpublished results), proved to be extremely stable. In homogenates stored at 0° and assayed at intervals, there was usually little loss of activity for 4–6 days, although in some preparations a 20–30 % decrease was observed after 3–4 days. To determine the effect of the stabilizing agents on the loss of fumarase activity, portions of homogenates were treated with succinate or lyophilized albumin extract or water. In homogenates whose activity had declined by 70 % after 10 days, the presence of either succinate (Table 10) or albumin extract completely prevented the loss of activity. In a recent experiment, fumarase has been found to be stable for at least a month in the presence of either stabilizing agent.

DISCUSSION

The literature contains a number of instances of the stabilization or protection of enzymic activities by various techniques such as the use of inert proteins, coenzymes, metals, reducing agents, chelators or the specific substrates of the enzymes concerned. Keilin & Hartree (1949) found that the impairment of heart succinoxidase activity which accompanied lyophilizing or repeated freezing at low temperatures could be restored by the addition of proteins or gelatinous phosphates to the test system. They ascribed the reactivation to the re-establishment of a suitable colloidal configuration in which the various components were so orientated that an enhanced mutual accessibility ensued. Similarly, Borei (1950) observed that in aged preparations of horse heart, cytochrome oxidase activity decreased on storage and could be re-activated through the addition of denatured globin or horse serum to the test system. Dialysis of the serum did not prevent the reactivation. Desiccation of the horse-heart preparations also lowered the initial cytochrome oxidase activity by 50 %, but if the desiccation was carried out in the presence of horse serum, the original activity was retained and the stability toward ageing was greater. Sacktor (1954) reported that in the absence of an inactive protein degenerative changes of housefly mitochondria began immediately after their isolation and lysis was complete within 30 min. The addition of crystalline bovine serum albumin to a phosphorylating system of fly mitochondria prevented the complete abolition of phosphate uptake and the partial inhibition of oxygen uptake. Watanabe & Williams (1953) found that crystalline albumin maintained the normal form and structure of insect sarcosomes for 24 hr. or longer. Crystalline serum albumin was also used by Polis & Shmukler (1957) to reactivate phosphorylation in aged (0°) rat-liver mitochondria; dialysis of the albumin did not alter the effect. Recently, Nason & Lehman (1956) showed that vitamin E activated both DPNH- and succinic-cytochrome *c* reductase of rat skeletal muscle but not cytochrome *c* oxidase. Vitamin E could be replaced by a lipid cofactor obtained from *iso*octane extracts of purified DPNH

cytochrome *c* reductase or by a lipid bound to crystalline bovine serum albumin. Peptone was reported to activate and stabilize diluted ox erythrocyte carbonic anhydrase (Scott & Mendive, 1941), but dialysis did not prevent these effects, and gelatin and casein were also good stabilizers of the enzyme.

The results presented here on the stabilization of a number of components of the electron-transport systems of *T. pyriformis* by a water-soluble, thermostable, diffusible material of blood serum apparently associated with the albumin fraction, or by succinate, citrate or *iso*citrate, appear to be different from the findings reviewed above as well as from many other observations on protection and stabilization of enzyme activities too numerous to mention here. In attempting to assess these results, it is helpful to reconsider some of the salient points that have been given. As the enzyme systems studied increased in complexity, they exhibited increased instability, but the loss of activity of each enzymic link could be prevented for varying periods by the same stabilizing agent(s). Whether the mode of action of these agents is by (1) replacement of a disintegrating substance whose function is to gear the components of the particulate electron-transport systems to each other, (2) prevention of this disintegration, or (3) maintenance of a particular range of osmotic and ionic conditions or by some other means, little can be said at this time. Some of these possibilities are being explored further.

There is no evidence at the moment to indicate that the stabilization by blood serum (or crude albumin), peptone or yeast is effected by its content of both succinate and citrate. In fact, it can be calculated that in the volume of serum required for stabilization of the succinoxidase system, the total amount (Albritton, 1952) of these acids is 25–50 times less than the succinate level which confers complete protection for 24 hr. Although it is conceivable that a group of physiologically occurring compounds, more or less structurally related to succinate, citrate and *iso*citrate, is responsible for the action of serum, the demonstrated marked specificity of the effective acids militates against this view. It is also possible that the action of serum (peptone, yeast) is due to some type of succinate-containing compound whose effect can be imitated by larger amounts of pure succinate.

Finally, it should be emphasized that the description of the labile electron-transport systems in the *T. pyriformis* preparations studied here does not necessarily imply that this is an inherent property of the systems studied in all types of homogenates and extracts of the organism. However, we have found that cell-free preparations made by freeze–thawing or sonic disruption also yield very unstable respiratory-enzyme activities.

SUMMARY

1. Homogenates of *Tetrahymena pyriformis* aged at 0°, or heated at 30°, lose overall succinoxidase, glutamic oxidase and β-hydroxybutyric oxidase activities rapidly. When different electron or hydrogen acceptors (dyes, diphosphopyridine nucleotide) are used in place of oxygen, stability increases, but only relatively. Previously, several reduced diphosphopyridine nucleotide-oxidizing enzymes assayed with oxygen, cytochrome *c* or a dye as electron acceptor were found to behave similarly.

2. Addition of a crude bovine blood-albumin preparation to fresh *Tetrahymena* homogenates which were then aged at 0°, or heated under mild conditions, stabilized the complete succinoxidase and reduced diphosphopyridine nucleotide oxidase systems, as well as succinic dehydrogenase and reduced diphosphopyridine nucleotide cytochrome *c* reductase, for 24 hr. or more. Crystalline bovine serum albumin and other proteins were incapable of stabilizing these enzymes, whereas horse, rat, human and chick serum, proteose peptone and yeast extract all protected against the loss of succinoxidase activity. Attempts to reproduce the action of the crude albumin extract by ageing homogenates at 0° with various compounds which have been implicated in electron-transport function or which occur in blood plasma were generally unsuccessful.

3. The slower decline of the succinoxidase activity of rat-liver homogenates aged at 0° could also be partly prevented by ageing in the presence of the crude albumin preparation.

4. The stabilizing material of the crude albumin preparation was shown to be water-soluble, diffusible and resistant to boiling at neutral or acid pH for short periods. An unheated or boiled lyophilized diffusate of the crude albumin extract stabilized the succinoxidase system of *Tetrahymena* homogenates aged for 24 hr. at 0°. Dialysis of rat serum, yeast extract and proteose peptone also abolished their ability to stabilize the succinoxidase system.

5. Under the experimental conditions described, the loss of succinoxidase activity was largely irreversible; the activity present at a given time after the preparation of homogenates was a function of the time of addition of the albumin extract to the homogenates.

6. Addition of sodium succinate to homogenates of *T. pyriformis* aged at 0° also prevented for at least 24 hr. the rapid loss of activity of the complete succinoxidase and reduced diphosphopyridine nucleotide oxidase systems. Moreover, the activities

of reduced diphosphopyridine nucleotide cytochrome *c* reductase, diphosphopyridine nucleotide-linked glutamic and β-hydroxybutyric dehydrogenases and fumarase were also stabilized for varying periods by ageing homogenates at 0° with succinate.

7. When succinate-supplemented homogenates were centrifuged and the resultant particulate preparations resuspended in water, the stabilization effect was not apparent unless additional succinate was added to the resuspended preparations.

8. Heat denaturation of succinoxidase, reduced diphosphopyridine nucleotide oxidase and succinic dehydrogenase activities was overcome by heating homogenates in the presence of succinate.

9. Of a large number of compounds, many of which are closely related to succinic acid structurally, only *iso*citrate and citrate (as well as succinylcholine and succinimide) gave appreciable protection of the succinoxidase and reduced diphosphopyridine nucleotide oxidase systems, indicating that the structural requirements of the succinate-stabilization effect are very rigid.

10. It is suggested that the present findings are different from previous observations on the stabilization and protection of enzymic activities by various methods, and the results are discussed.

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The Absorption of Cystine and Cysteine from Rat Small Intestine

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Although there have been many reports in recent years of experiments designed to study the rates and mechanisms of the absorption of amino acids from the small intestine, cystine and cysteine do not appear to have been included in the compounds studied. This is perhaps due in part to the relative insolubility of cystine, and the fact that one of the more elegant techniques at present being used for such studies involves the use of isolated everted sacs (Wilson & Wiseman, 1954) of small intestine, which cannot be used for the study of the absorption of cysteine. This is because the oxidation of cysteine in oxygenated solution is rapid, and anaerobic conditions inhibit absorption processes involving 'active transfer' (Wilson & Wiseman, 1954) in this preparation.

In the work reported here cystine absorption has been studied with both the everted-sac technique and a lumen-perfusion technique *in situ*, whereas the absorption of cysteine has been studied with the latter only.

Hess (1949) has briefly reviewed earlier work in the absorption of cystine, which, however, had not been concerned with the elucidation of the nature of the absorptive process, but only with the overall rates of absorption after ingestion by animals of the DL- and L-isomers of the amino acid. In his own experiments, in which he fed rats by stomach tube with solutions of the sodium salts of cystine isomers and of L-cysteine, Hess (1949) found no significant differences between the rates of absorption of L-, D- or DL-cystine. *meso*Cystine was absorbed at a lower rate than the other isomers. L-Cysteine was absorbed more slowly than the L-, D- and DL-isomers of cystine. A preliminary report of the work described here was given by Neil (1957).

MATERIALS AND METHODS

Animals. Laboratory stock male albino rats weighing 200–300 g. were used throughout.

Chemicals. L-Isomers of cystine, cysteine and methionine were laboratory-reagent grade (British Drug Houses Ltd., Poole, Dorset); D-isomers of cystine CFP (California Foundation Purity) and cysteine HCl CFP were obtained from the California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A. Other compounds used were AnalaR, Polaritan (Hopkins and Williams Ltd.), or laboratory-reagent grade (British Drug Houses Ltd.).

Lumen-perfusion technique in situ. Rats were fasted for about 12–15 hr. and then anaesthetized with 50% (w/v) urethane solution by injection of 0·9 ml. intraperitoneally and 3 ml. subcutaneously/kg. body wt. If necessary, surgical anaesthesia was induced with ether just before dissection. Tracheal cannulation was always used. Dissection, intestinal cannulation and perfusion were by conventional techniques. The perfusion pressure measured at the proximal end of the perfused segment was kept at 9–11 cm. water throughout each experiment. The volume of perfusion fluid [Krebs & Henseleit (1932) bicarbonate saline solution omitting calcium and glucose ('bicarbonate saline')] was about 55 ml. Samples of 1 ml. were removed periodically for analysis and solution was added to the fluid reservoir to maintain constant volume. At the end of the perfusion period the segment of gut was removed, slit open longitudinally, and blotted gently but firmly between sheets of filter paper and weighed immediately. When cysteine was being studied the solution was equilibrated with N_2+CO_2 (95:5); in all other experiments O_2+CO_2 (95:5) was used.

Everted-sac technique (in vitro). This preparation was set up according to Wilson & Wiseman (1954). Slight modifications were introduced. Rats were anaesthetized and the 'eversion rod' was tied into the gut while the latter was still perfused with blood. The segment was then rapidly dissected free from mesentery and placed in a bath of bicarbonate saline at room temperature [gassed with O_2+CO_2 (95:5)], and eversion and isolation of the sacs were carried out in this bath. Incubation was at 37°. A fine sintered-glass gas bubbler was placed just beneath the sacs, which were gently bathed in the stream of fine bubbles of O_2+CO_2 (95:5). After incubation the sacs were removed from the bath, and gently blotted and the contents removed for analysis. The empty sacs were then treated as described above (at the end of the perfusion period).

Analytical methods

Water content, fat extraction and potassium analysis. The water contents of segments were obtained by drying the tissue at 105° to constant weight. Fat was extracted from the dried material by the method of Hastings & Eichelberger (1937). The fat-free dry residues were dissolved in nitric acid and the potassium concentrations in suitable dilutions determined by flame photometry. Both water and potassium contents were calculated on a fat-free-tissue basis. Where mucosal cells alone were required the sacs were slit open, gently pressed between filter paper, and dissected free from fat on a cold table and the mucosal cells carefully scraped from the muscle layers with a blunt spatula. Fat was not extracted from the dried residues of the cells before dissolving in nitric acid for potassium analyses.

Cystine and cysteine. These compounds were determined polarographically (Brdicka, 1933, 1938) with Tinsley polarographic equipment. The supporting buffer was made by mixing 1 vol. of aq. 0·125M-NH_3 soln. with 2 vol. of 0·125M-NH_4Cl soln., and then adding 2 ml. of saturated Na_2SO_3 soln./100 ml. of the mixture. To 3·9 ml. of this buffer were added $(1-x)$ ml. of water and x ml. ($\leqslant 1$ ml.) of solution containing 1–3 μg. of cystine (or cysteine). Immediately before the estimation 0·1 ml. of 0·1M-$CoCl_2$ soln. was added.

The contents of the sacs were found to contain protein, which interfered with this determination. To avoid this, 0·5 ml. of the solution from the sac was mixed with 0·5 ml. of 10% (w/w) $HClO_4$, and allowed to stand for a few minutes and then centrifuged. A suitable dilution of the supernatant was used for polarography. All determinations were carried out at least in duplicate.

As the polarographic method does not differentiate cystine and cysteine, it was necessary when studying the absorption of cysteine to use a method whereby the contents of cystine and cysteine could be obtained separately in the same solution. Cystine plus cysteine was determined polarographically, and then cystine alone after allowing the cysteine to react with iodoacetate. Cysteine was found by difference. A volume (0·5 ml.) of solution containing cysteine (0·04–0·4 mg.) was mixed with excess of 0·016M-iodoacetate (0·1–0·8 ml.) and 2 drops of aq. 5N-NH_3 soln. were added. The mixture was heated for 2 min. in a boiling-water bath, and cooled and diluted to 10 ml. This solution, or a suitable dilution, was used for polarographic analysis.

S-Carboxymethylcysteine was prepared by the method of Dickens (1933), and separated quantitatively from traces of cystine by electrophoresis. This compound, which is formed by the reaction between iodoacetate and cysteine, appears to be reduced at the dropping-mercury cathode. No sharp reduction potential was observed. However, the diffusion current in the region of the cystine catalytic-curve peak is very small under the conditions used and no correction was necessary. The peak of the cystine catalytic curve could almost invariably be accurately delineated, but the base line was not always so clear. In view of this difficulty the accuracy of the method was probably not greater than ±7% (outer limits).

Methionine. This was estimated by the method of McCarthy & Sullivan (1941).

Glucose. This was estimated by the method of Nelson (1944).

Histology. Samples of normal and perfused (or incubated) gut were fixed in formol-saline, and embedded and sections stained with haematoxylin and eosin.

RESULTS

Lumen-perfusion experiments in situ

Preparation control. (*a*) Potassium and water contents. In perfused segments of duodenum, jejunum and ileum the potassium content of the whole tissue remained practically normal. The water content of such segments was about 3% higher than that of control samples. Results of these analyses are shown in Table 1.

(*b*) Histology. Apart from slight oedema the mucosal epithelium was practically normal in sections from perfused segments.

(*c*) Glucose absorption. The well-established inhibition of active absorption of glucose by phlorrhizin in such preparations was used as a functional test. The inhibition was unequivocally demonstrated.

Absorption of cystine and cysteine. The absorption of cystine and cysteine was studied by following the decrease in amino acid concentration in the perfusion fluid. Solution containing cystine was prepared either by shaking with excess of solid cystine, followed by filtration, or by dissolving cystine in a small excess of sodium carbonate solution and adding this solution immediately to 'bicarbonate saline' through which O_2+CO_2 (95:5) was being passed. Allowance was made for the bicarbonate formed from the sodium carbonate when the solution was prepared. No racemization of L-cystine occurred during this procedure. One advantage of the preparation *in situ* over the everted-sac technique is that because the gut in the former is oxygenated by the blood flow it is possible to keep the perfusion fluid O_2-free. Cysteine was found to remain almost completely unoxidized in the perfusion *in situ* when N_2+CO_2 (95:5) was used for the gas lift and in the spaces above the fluid level in the reservoir.

The relations found between the rates of jejunal absorption of L-cystine and L-cysteine and the initial concentration of the amino acids in the perfusion fluid are shown in Figs. 1 and 2. In each case the relation was approximately linear, although there was some scatter of the results, especially at high initial concentrations. In the concentration ranges over which comparable results were obtained, the rate of absorption of L-cystine was found to be about 70% of that of L-cysteine. Substitution of N_2 for O_2 in the cystine-absorption experiments did not affect the rate of absorption of this amino acid. Estimations of both cystine and cysteine were performed on each sample taken during cysteine-absorption experiments.

Table 2 shows the results of experiments in which the rates of absorption of the two amino acids were studied in various regions of the small intestine. The regions used were proximal duodenum, mid-jejunum and distal ileum. The rates of absorption of cystine clearly decreased in the order duodenum, jejunum, ileum, with a small overlap between the ranges observed for duodenum and jejunum. The rate of absorption of L-cysteine is also lower from the jejunum than from the duodenum.

Table 1. *Potassium and water contents of rat small intestine*

Normal and *in situ* lumen-perfused samples. Potassium contents are calculated on a fat-free dry-tissue basis, and water on fat-free fresh tissue. Standard deviations (S.D.) have not been calculated where less than five results were available.

Experiment	No. of experiments	Potassium, m-equiv./kg. Mean	Potassium, m-equiv./kg. S.D. or range	Water (%, w/w) Mean	Water (%, w/w) S.D. or range
		Controls			
Duodenum	5	435	21	78·8	0·97
Jejunum	17	420	22	78·7	0·97
Ileum	1	417	—	78·3	—
		Perfused			
Duodenum	16	457	24	81·7	0·93
Jejunum	20	448	23	81·5	1·36
Ileum	4	448	443–453	82·1	81·2–83·9

Table 2. *Absorption of* L-*cystine and* L-*cysteine from various regions of the rat small intestine* in situ

Region of gut	No. of experiments	Mean initial amino acid concentration (mM)	Mean absorption rate (μmoles/g. of fat-free dry tissue/hr.)	Range of rates
		L-Cystine		
Duodenum	5	0·875	75·0	57·1–92·5
Jejunum	8	0·821	47·5	36·7–58·8
Ileum	4	0·872	25·9	22·9–31·7
		L-Cysteine		
Duodenum	3	1·77	222	155–268
Jejunum	3	1·77	150	132–166

The presence of 0·3 mM-2:4-dinitrophenol (DNP) in the perfusion fluid had no influence on the absorption of D-glucose, L-cystine (Fig. 3) and L-methionine. The presence of DNP did not alter the potassium and water contents of the perfused segments. This is in contrast with the large loss of intracellular potassium and increase in water observed when everted sacs were incubated in bicarbonate saline containing DNP (0·3 mM) (Table 6).

The D-isomers of both cystine and cysteine were absorbed from rat duodenum at much lower rates than their L-isomers. Table 3 shows the results obtained. Comparison of the ranges of results obtained shows no overlap between the rates for L- and D-isomers in either case. These results provided

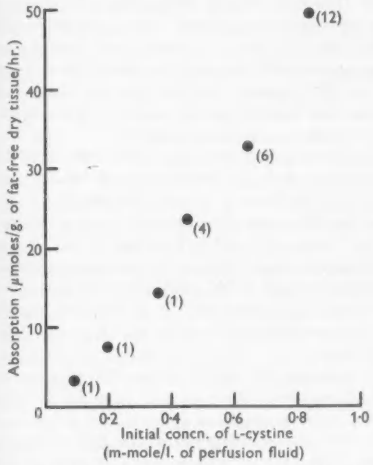

Fig. 1. Relation between the rate of absorption of L-cystine and the initial concentration in the perfusion fluid (*in situ* technique). Figures in parentheses are numbers of results, which were averaged to yield each point. Rates were calculated over 90 min. perfusion periods.

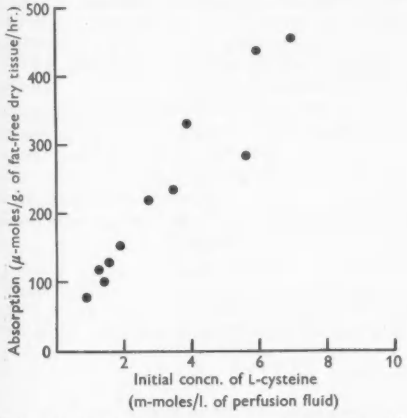

Fig. 2. Relation between the rate of absorption of L-cysteine and the initial concentration in the perfusion fluid. Points represent individual experiments. Rates were calculated over 90 min. perfusion periods.

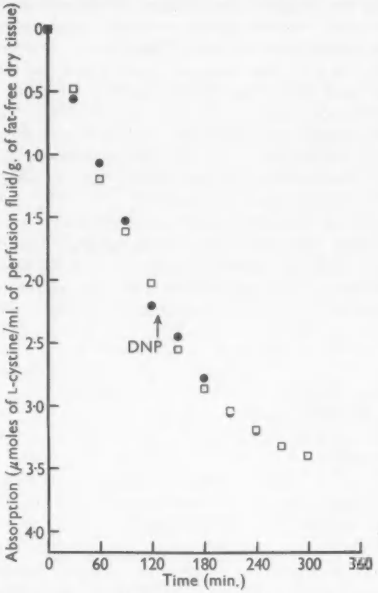

Fig. 3. Absorption of cystine from rat jejunum (*in situ* technique). □, L-Cystine alone; ●, control period followed by injection of DNP (to make 0·3 mM). Starting concentrations, volumes of perfusion fluid and weights of the fat-free dry segments for the two experiments were within 5% of each other.

Table 3. *Rates of absorption of* D- *and* L-*isomers of cystine and cysteine from rat duodenum* in situ

Number of experiments is given in parenthesis. Values given are absorption rates calculated from 90 min. perfusion periods. Starting concentrations of amino acids in perfusion fluids were similar for the experiments with D- and L-isomers in each pair.

	Mean absorption rates (μmoles/g. of fat-free dry tissue/hr.)	
	L-Isomers	D-Isomers
Cystine	80 (5)	27 (4)
Cysteine	222 (3)	57 (3)

preliminary evidence that an active process was involved in the absorption mechanism for the L-isomers in each case.

Everted-sac experiments in vitro

Preparation control. (*a*) Potassium and water contents. At the end of incubation for 1 hr. at 37° in bicarbonate saline, the intestinal sacs were found to have lost about 10% of their normal potassium content. The water content rose from about 78·5% to about 84% (Table 4). Before using the preparation further, it was necessary to find out whether these changes represented a continuous deterioration of the tissue or whether a new steady state was set up in which the potassium and water contents remained constant, but at levels different from those of the normal tissue.

Five sacs were therefore prepared from a single length of jejunum and the whole tissue was analysed after various times of incubation. Fig. 4 shows the results of such an experiment.

Loss of potassium and accumulation of water evidently commenced immediately after dissection of the segment. Small changes were found to have occurred by the time eversion was complete, a matter of about 2–4 min. after the start of dissection. The main changes occurred between eversion and the end of the first 10 min. of incubation. Thereafter both the potassium and the water contents of the whole tissue remained relatively constant.

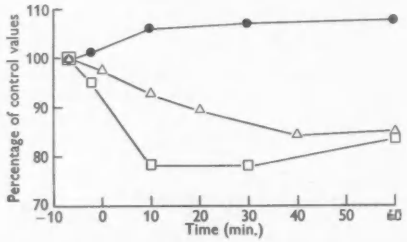

Fig. 4. Variation of potassium and water contents of everted-sac tissue with time. Incubation was in bicarbonate saline; 37°; O_2+CO_2 (95:5); 1 hr. Negative values on the abscissa represent times between cutting off intestinal blood supply (at about −7 min.) and start of incubation (at zero time). □, Potassium and ●, water contents of whole tissue; △, potassium contents of mucosal cells. All ordinate values are expressed as percentages of the appropriate normal control values.

Further experiments were carried out to determine whether these changes involved the mucosal cells. The rate of loss of intracellular potassium from these cells was found to be more gradual than that from the whole tissue, a new steady state being established between 20 and 40 min. of incubation. The potassium content of the mucosal cells at the end of incubation for 1 hr. was about 85% of the control value (Fig. 4).

(*b*) Histology. Comparisons of the normal and incubated tissues showed that there was a certain amount of oedema of the epithelium in the latter. This was more pronounced in the cells nearer the tips of the villi. The mucosal cells originally covering the tips of some of the villi appeared to have been sloughed off. The cells within the crypts were practically normal. It is possible that cells were stripped from the villi tips during the eversion procedure.

(*c*) Transfer of L-methionine. The transfer of L-methionine from the mucosal side to the serosal side of both hamster and rat small intestine has been shown to involve an active transport mechanism (Wilson & Wiseman, 1954; Wiseman, 1955). The absorption of this substance was considered particularly suitable as a control functional test in the present case. Concentration gradients of a similar magnitude to those found by Wiseman (1955) were observed.

Conclusions from the preparation controls. (*a*) and (*b*) above indicate that the physiological state of the rat small intestine was not as well preserved in the everted-sac technique as in the lumen-perfusion technique *in situ*. However, the establishment of a new steady state observed in (*a*) above, and the creation of concentration gradients in the experiments with L-methionine appeared to indicate that the tissue was still functional. It was decided therefore to use the preparation for further studies on the nature of the transport of cystine across the rat-intestinal wall, although bearing in mind the limitations of the technique.

Table 4. *Control experiments on everted sacs of rat jejunum*

Calculation of potassium and water contents are as for Table 1. Sacs were incubated in bicarbonate saline; 37°; O_2+CO_2 (95:5); 1 hr. Number of experiments is given in parentheses.

	Potassium (m-equiv./kg.)		Water (%, w/w)	
	Mean	S.D.	Mean	S.D.
Normal tissue	420	22 (17)	78·7	0·97 (17)
Sacs incubated in bicarbonate saline alone	393	14 (6)	83·6	0 (3)
Mean values for all incubated jejunal sacs	382	23 (23)	84·3	0·58 (19)

Cystine transfer. Everted sacs of rat small intestine were prepared and incubated in bicarbonate saline containing cystine. The initial cystine serosal/mucosal concentration ratio was 1·0. After 1 hr. the ratio was redetermined. Table 5 illustrates the results obtained with the L- and D-isomers of cystine. It is evident that L-cystine was transported from the mucosal side to the serosal side of the sacs against a concentration gradient. No such transport of D-cystine occurred. The reasons for the decrease in the serosal/mucosal concentration ratio in the latter case was almost certainly due to the transport of water from the mucosal to the serosal side (Wilson & Wiseman, 1954).

The effect of DNP on the preparation was also studied, since the inhibition of absorption processes by this substance has been cited as evidence in favour of the indirect involvement of 'a phosphorylation process' in active transport mechanisms (Fridhandler & Quastel, 1955). Jejunal sacs were incubated in bicarbonate saline alone and in bicarbonate saline containing 0·3 mM-DNP. At the end of 1 hr. the sacs were briefly examined macroscopically and the potassium and water contents determined. Similar experiments were carried out in which, at the end of the incubation period, the mucosal cells were gently scraped off the muscle layers and analysed separately. Table 6 shows the results obtained. It is clear that DNP caused a severe loss of intracellular potassium and an increased accumulation of water in the gut. The total potassium content of the mucosal cells alone was reduced to about 50 % of the normal level.

Macroscopic examination of the tissue after exposure to solution containing DNP revealed a curious 'granular' appearance of the mucosa. The tissue had the appearance of having grossly deteriorated, and the potassium and water analyses confirmed this conclusion. In view of the gross generalized damage to the tissue caused by the presence of DNP in the solution, further experiments planned to study the effects of DNP on cystine transport with this preparation were abandoned.

Table 5. *Demonstration of active transport of* L-*cystine by rat small intestine*

Sacs were incubated in bicarbonate saline; 37°; $O_2 + CO_2$ (95:5); 1 hr.

Region of small intestine	No. of sacs	Initial concn. (mM)	Mean concn. gradient after 1 hr.
	L-Cystine		
Duodenum	2	0·62	2·38
Jejunum	3	0·85	2·90
Ileum	2	0·62	1·57
	D-Cystine		
Jejunum	3	0·77	0·84
Jejunum	3	0·80	0·93

DISCUSSION

The two rat-intestine preparations used in these studies differed considerably from one another both in the condition of the tissue during the experiments and in the factors which affected the rates of transfer of metabolites across the gut wall.

The intestinal tissue in the lumen-perfusion technique appeared to remain practically normal, as judged by the potassium and water contents and histological examination, during the period of the experiments. The everted-sac technique, however, caused marked changes in the tissue, by the same criteria. Even so, it was clear that the sac tissue was not deteriorating at an appreciable rate after the establishment of a new steady state. It was concluded therefore that both preparations could be used for absorption studies, although recognizing clearly that the states of the tissues differed from each other, and from normal tissue, at least in the respects revealed by the control tests.

The absorption routes of metabolites were different in the two preparations. In the technique *in situ* the route approximated to normal, but in the everted-sac technique the amino acids had to traverse the whole intestinal wall. However, the conditions were more reproducible in the technique *in vitro* than in the technique *in situ*, because the latter was subject to such factors as variation in the blood flow perfusing the gut capillaries, amounts of anaesthetic required, degree of shock (Guthrie & Quastel, 1956) and degree of distension of the segment during perfusion.

Table 6. *Effect of* 2:4-*dinitrophenol on incubated sacs of rat jejunum*

Mucosal cells were removed after incubation of the whole preparation. Sacs were incubated in bicarbonate saline; 37°; $O_2 + CO_2$ (95:5); 1 hr. Calculations of potassium and water contents for *A* were as for Table 1. Mucosal cells were not fat-extracted.

Experiment	No. of sacs	Potassium (m-equiv./kg.)	Water (%, w/w)
A. Whole preparation			
Bicarbonate saline alone	3	395	83·6
Bicarbonate saline + DNP (0·3 mM)	3	215	85·2
B. Mucosal cells			
Bicarbonate saline alone	3	436	—
Bicarbonate saline + DNP (0·3 mM)	2	224	—

These points are emphasized because it is important to compare the results of absorption studies in relation to the techniques used. This is illustrated below, where the disparity between the present results and those of Hess (1949) is considered.

The approximately linear relations between rates of absorption and concentration of both L-cystine and L-cysteine, which were observed with the technique *in situ*, do not exclude 'active-absorption' mechanisms. If, as suggested by Wiseman (1955) for the hamster small intestine, there exists a common mechanism for absorption of α-monoaminomonocarboxylic acids it seems likely that the capacity of this system would be large. If L-cystine is absorbed by this same mechanism the concentration required to 'saturate' it might well be far beyond the highest concentration limit imposed by the solubility of the amino acid (about mM). In this case there would be no practical range over which the rate of absorption would become independent of the concentration. The upper limit of concentration used in the experiments on L-cysteine was about 850 mg./l. (7 mM). This was also apparently too low to 'saturate' the active mechanism if, as is suggested by the later experiments, L-cysteine is actively absorbed.

Fisher & Parsons (1949), using an isolated loop of rat intestine *in vitro*, showed that the rate of absorption of glucose decreased with increasing distance from the pyloric sphincter. The present experiments indicate that a similar phenomenon occurs with L-cystine and L-cysteine. However, this may in part be due to variation in blood flow, and therefore in amino acid clearance, in different regions of the intestine in the technique *in situ*. It is conceivable also that the rates of absorption, especially of amphoteric molecules, would vary appreciably with changes in pH of the fluid bathing the lumen of the intestine. *In vivo* there is a considerable pH gradient along the small intestine during digestion and absorption, whereas in the experiments reported here absorption rates were determined in various regions only at pH 7·4.

The results obtained with D- and L-isomers of both amino acids with the technique *in situ* strongly suggest active absorption mechanisms. The results of experiments with D- and L-cystine in the everted-sac technique support this conclusion.

The reasons for the disparity between the findings reported here and those of Hess (1949) are not clear. In the feeding experiments *in vivo* of Hess (1949) the sodium salts of the amino acids, which are alkaline in aqueous solution, were used and it is difficult to know what effect such solutions would have on the mucosa of the stomach and small intestine before the pH had been reduced by the secretions of the alimentary canal. Further, there is the possibility that the amounts of cystine isomers (about 100–300 mg.) fed to the rats as sodium salts (the volumes of the solutions were not given) would lead to precipitation of free cystine in the gut if the pH was reduced below about 8. Such an occurrence would make the interpretation of the results very difficult.

SUMMARY

1. The absorption of isomers of cystine and cysteine from the small intestine of the rat has been studied with a lumen-perfusion (*in situ*) and an everted-sac technique.

2. Tissue potassium and water contents, histological examination and application of functional tests have been used to assess the condition of the intestinal tissue in the preparations.

3. The rates of absorption of both L-cystine and L-cysteine were found to increase approximately linearly with increasing concentration. The rates of absorption from duodenum, jejunum and ileum were found to decrease in that order.

4. The absorption of L-cystine but not D-cystine has been shown to be an active process. Evidence is presented which shows that a similar process is probably also involved in the absorption of L-cysteine.

5. Previous work which demonstrated no difference between the rates of absorption of the D- and L-isomers of cystine is discussed in the light of the experiments reported here.

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The Carbohydrate Complexes of Bronchial Secretion

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The mammalian respiratory tract maintains resting levels of secretion which are subject to the external environment and also to nervous stimuli from within (Florey, Carleton & Wells, 1932). The secretion from the bronchial mucosa of the healthy human subject is, however, rarely available for investigation as normally it is swallowed together with the secretions of the upper respiratory tract and bucco-pharynx. In contrast, sputum, which is the pathological counterpart of bronchial secretion, is relatively easy to obtain, particularly during winter, in sufficient quantities for study. None the less, no substantial work on the composition of the mucous ground substance of sputum has been done since the original work of Müller (1896).

In this Laboratory, interest in the mucin of sputum was stimulated by the work of Himmelweit (1948), who found the mucin to contain a carbohydrate complex (F. Himmelweit, personal communication) which was an inhibitor of influenza-virus haemagglutination; his work has been confirmed by Curtain, Marmion & Pye (1953). The present paper describes the isolation, purification and properties of the carbohydrates contained in pooled sputa. A comparison of the properties of one of these carbohydrates with those of a similar mucoprotein occurring in urine is also made.

MATERIALS AND METHODS

Sputum. Sputum samples were collected from patients who were suffering from long-standing inflammatory conditions of the lung, such as chronic bronchitis. The samples, which were stored in a refrigerator at 0–4°, were sorted each week and only those which were either 'mucoid' or faintly 'muco-purulent' were retained. The terms 'mucoid' and 'muco-purulent' are clinical assessments of sputum based on its gross appearance (Chamberlain, 1957). 'Mucoid' sputum is either colourless or white and has a gel-like consistency. 'Muco-purulent' sputum has the same consistency as 'mucoid' sputum but has a yellow or green tinge due to the admixture of pus, the colour of the sputum depending largely on the amount of pus present. The selected samples were pooled, washed with water and dried with acetone. The resulting grey-white powder, termed acetone-dried sputum, was used for this investigation. A total of 1216 specimens was collected, of which 385 were selected for use; the volume of sputum obtained from the chosen samples was 1·925 l., which in turn yielded 28·6 g. of acetone-dried sputum.

Urine. Urine from normal males was collected in 2·5 l. bottles. Chloroform (10 ml./l.) was added and the bottles were stored in a refrigerator at 0–4°.

Total nitrogen. This was estimated by an adaptation of the Markham (1942) steam-distillation technique, followed by colorimetric determination of the nitrogen as ammonia by the nesslerization procedure of Koch & McMeekin (1924). Combustion of the samples was carried out with the copper–selenium reagent of Campbell & Hanna (1937). A.R. $(NH_4)_2SO_4$ and thrice-recrystallized glucosamine hydrochloride were used as standards.

Phosphorus. Organic and inorganic phosphorus were determined by the modified Fiske & Subbarow (1925) method described by Kabat & Mayer (1948).

Reducing sugars. Estimation of reducing sugars was carried out by the method of Somogyi (1945) and is expressed in terms of glucose.

Amino sugars. The procedure devised by Schloss (1951), which is based on the method of Elson & Morgan (1933), was used to estimate amino sugars in terms of glucosamine. Schloss (1951) recommended that the complex which is formed when a solution of glucosamine is treated with acetylacetone and coupled with *p*-dimethylaminobenzaldehyde, in the manner he describes, should stand at 30° for 24 hr. This incubation allows the absorption spectrum of the glucosamine complex, which initially has a maximum at 530 mμ, to change to a more stable pattern with a maximum at 512 mμ. It was found, however, that the glucosamine complex attained a stable absorption spectrum with a maximum at 512 mμ if the solution stood at 22·5° for 24 hr.

Partition chromatography. The ascending-chromatographic technique described by Williams & Kirby (1948) and Bray, Thorpe & White (1950), with Whatman no. 4 paper, was used for sugars. The hydrolysates of the mucopolysaccharides and of the mucoproteins were prepared for chromatography by the following modification of the method of Malpress & Morrison (1949). The hydrolysates, which were dried *in vacuo* over H_2SO_4 and NaOH, were extracted at 60° with 0·4 ml. amounts of dry pyridine. The resulting solutions were filtered by suction through Pasteur pipettes, the ends of which had been constricted and plugged with filter-paper pulp, and the filtrates were again dried *in vacuo*. The process was repeated several times to ensure maximum de-ionization of the hydrolysates. The solvent systems used for irrigation were as follows: butanol–acetic acid–water (40:10:50, by vol.); pyridine–butanol–benzene–water (30:50:10:30, by vol.); collidine–water; phenol–water. Development was carried out with aniline hydrogen phthalate and the silver nitrate reagent of Trevelyan, Procter & Harrison (1950). Two-dimensional chromatography was used to identify the amino acids in the mucopolysaccharides, by the method of Consden, Gordon & Martin (1944). The papers were irrigated with the butanol–acetic acid–water and the pyridine–butanol–benzene–water mixtures described above. Ninhydrin was used to show the amino acid spots.

pH. This was determined with a Cambridge pH meter and a saturated KCl soln.–calomel/glass electrode system.

Viscosity. The modification of the Ostwald viscometer described by Cannon & Fenske (1938) was used to determine the viscosity of 0·2% solutions of the mucopolysaccharides and mucoproteins in 0·85% NaCl soln. and in water. The measurements were carried out at 25±0·01°. The results were expressed as the relative kinematic viscosity (ν) relative to that of the solvent in which the carbohydrates were dissolved.

Colorimetric and spectrophotometric measurements. These measurements were carried out on a Hilger Spekker photoelectric absorptiometer and on a Beckman quartz spectrophotometer.

Electrophoresis. A Perkin–Elmer Tiselius apparatus was used.

Urinary mucoprotein. This complex was isolated from the collected urine by a modification of the method of Tamm & Horsfall (1952). The material when freshly prepared was completely soluble in 0·025M-Na_2HPO_4–NaH_2PO_4 buffer, pH 6·8, but after a lapse of 3 months it was found to be only partly soluble in this buffer. The mucoprotein was found to be electrophoretically homogeneous.

EXPERIMENTAL AND RESULTS

Isolation of the mucopolysaccharides and the mucoprotein

Digestion with proteolytic enzymes, originally employed by F. Himmelweit (personal communication) to extract the virus haemagglutinating inhibitory complex from acetone-dried sputum, was used here as the first stage of the isolation procedure. Pepsin was adopted as the enzyme of choice as it resolved the acetone-dried sputum into a supernatant, which contained the mucopolysaccharides, and an insoluble component which was largely composed of the mucoprotein. Incubation with the enzyme was carried out for 40 hr. It was found unnecessary to prolong the time of incubation to the periods of days and weeks that were employed by Kabat, Bendich, Bezer & Beiser (1947) to isolate blood-group mucopolysaccharides from sources such as saliva.

Several methods of purifying the mucopolysaccharides obtained from peptic digestion of acetone-dried sputum were tried; fractionation with 90% (w/v) phenol–water by the technique of Morgan & King (1943) gave the most satisfactory results. The details of the procedure used for isolating the mucopolysaccharides and the mucoprotein are given below.

Enzymic digestion. Acetone-dried sputum (5 g.) was dissolved in 1 l. of water at 0° and the solution was adjusted to pH 2·3 with 0·1N-HCl. Armour crystalline pepsin (50 mg.) was added and the mixture was incubated at 37° for 40 hr. under a layer of toluene. At the end of this time, the mixture was cooled and centrifuged at 0° to remove the insoluble fraction and the toluene.

Isolation of the mucoprotein. The insoluble fraction was washed repeatedly with water acidified with HCl to pH 2·3. It was then washed with ethanol and ether and dried *in vacuo* over P_2O_5; 620 mg. of grey–white powder was obtained. The bulk of this material was dissolved in water, and dialysed at 0° against water for 14 days and centrifuged at 10 000 *g* to remove insoluble residue. The mucoprotein contained in the resulting solution was dried from the frozen state. A voluminous white powder (350 mg.) was obtained.

Isolation of the mucopolysaccharides. The supernatant liquid from the peptic digestion was made 0·1M with respect to sodium acetate, and adjusted to pH 8·0 with 0·1N-NaOH and the mucopolysaccharides were precipitated with 2 vol. of ethanol at 0°, precautions being taken to obviate a rise in temperature due to the mixing of ethanol and water. The precipitate was redissolved in water and dialysed against water at 0° to reduce the large inorganic phosphorus content (Table 1), which would have interfered with the subsequent phenol

Table 1. *Composition of the mucopolysaccharides and the mucoprotein of sputum and of these complexes during the various stages of their isolation from acetone-dried sputum*

Analyses of the complexes were carried out on samples which were dried at 56° for 1 hr. The analytical results are expressed as a percentage of this dry weight. For methods of analysis, see text.

Preparation	Yield (%)*	Total N (%)	Amino sugars (%)	Reducing sugars (%)	Total P (%)	Inorganic P (%)	Organic P (%)
Acetone-dried sputum	—	10·4	10·8	18·7	0·62	—	—
Complex from peptic digestion insoluble at pH 2·3 (mucoprotein)	12	8·7	14·5	26·2	0·78	0·02	0·76
Complex from peptic digestion soluble at pH 2·3 (mucopolysaccharides)	25	5·9	20·4	38·3	1·13	1·06	0·07
Mucopolysaccharides after dialysis	22	6·1	22·5	45·8	0·33	0·25	0·08
Phenol-insoluble mucopolysaccharide	10	5·2	28·4	57·6	0·12	0·04	0·08
Phenol-soluble mucopolysaccharide	2	5·7	29·9	57·5	0·12	0·04	0·08
Mucoprotein	7	8·7	14·3	26·3	0·75	0·01	0·74

* The percentage yield was computed on a w/w basis with the parent acetone-dried sputum.

fractionation. The mucopolysaccharides were then recovered by ethanol precipitation as described above, and the precipitate was dried *in vacuo* over P_2O_5. This product (0·85 g.) was fractionated with 90% (w/v) phenol–water in a manner similar to that described by Morgan & King (1943) and Annison & Morgan (1952*a*, *b*). The procedure yielded a phenol-insoluble mucopolysaccharide, and also a phenol-soluble mucopolysaccharide which was isolated from the 90% phenol–water mixture by precipitation with ethanol at 10% of ethanol by vol. These two complexes were dissolved in water, and dialysed for 7 days against water at 0° and dried from the frozen state. There was obtained 394 mg. of the phenol-insoluble mucopolysaccharide and 84 mg. of the phenol-soluble mucopolysaccharide. Table 1 gives the results of the isolation of the mucopolysaccharides and the mucoprotein.

Properties of the mucopolysaccharides and the mucoprotein

Physical properties. The relative viscosity (ν) of a 0·2% solution of the phenol-soluble mucopolysaccharide in aq. 0·85% NaCl soln. at 25° was found to be 1·32. The relative viscosity of a 0·2% solution of the phenol-insoluble mucopolysaccharide, measured under the same conditions, was 1·51, and that of an 0·2% solution of the mucoprotein in aq. 0·85% NaCl soln. was 1·61.

Electrophoresis of the phenol-insoluble mucopolysaccharide and of the mucoprotein was carried out in a 0·02 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4, containing 0·15 M-NaCl at a potential difference of 105 V and a current of 20 mA. There was not enough of the phenol-soluble mucopolysaccharide for this determination. The ascending electrophoretic pattern of the phenol-insoluble mucopolysaccharide, photographed after 3 hr. (Fig. 1*a*), shows clearly that two components were present in a proportion of approximately 2:3. In contrast, the ascending pattern of the mucoprotein (Fig. 1*b*) recorded at 75 min. shows a single sharp boundary, indicating that the material is essentially electrophoretically homogeneous. Similar results were obtained at 2 hr. and at 3 hr. Both the electrophoretic pattern of the phenol-insoluble mucopolysaccharide and that of the mucoprotein showed a higher δ-boundary peak than might have been expected. This may represent in each case a slow-moving or stationary component of electrically near-neutral character at this pH. After one of the electrophoretic runs on the mucoprotein, this component was removed from the cell and its ultraviolet-absorption spectrum curve determined. This was found to be characteristic of a complex of protein nature and to show a peak at 277 mμ.

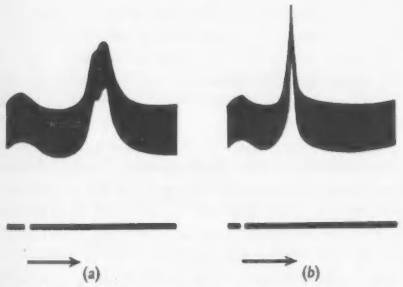

Fig. 1. (*a*) Ascending electrophoretic pattern of the phenol-insoluble mucopolysaccharide, scanned at 180 min., showing the two components of this complex. (*b*) Ascending electrophoretic pattern of the sputum mucoprotein, scanned at 75 min., showing the single sharply defined boundary. Both the electrophoretic runs were carried out in 0·02 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4, containing 0·15 M-NaCl, at 100 V and 20 mA. The concentration of the phenol-insoluble mucopolysaccharide was 2% (w/v) and that of the mucoprotein was 0·7%. The arrows indicate the direction of migration of the boundaries.

Chemical properties. Aqueous solutions of both the mucopolysaccharides and the mucoprotein were of neutral pH. Lowering the pH of solutions of the mucoprotein below 3·3 with 0·1 N-HCl caused the complex to precipitate; addition of N-HCl to a final concentration of 0·5 N-HCl caused it to redissolve. Neither the phenol-insoluble mucopolysaccharide nor the phenol-soluble mucopolysaccharide was precipitated from aqueous solutions by lowering the pH, or by boiling, or by the addition of equal volumes of reagents that normally precipitate proteins.

The presence of alkali-labile *N*-acetylhexosamine linkages was demonstrated in all three preparations by the method of Morgan & Elson (1934). The red solutions obtained from the two mucopolysaccharides were examined spectrophotometrically, and two absorption maxima at 548 and 588 mμ were found. These observations are in accord with those reported by Aminoff, Morgan & Watkins (1952) for the action of alkali on specific blood-group mucopolysaccharides.

When the phenol-insoluble and the phenol-soluble mucopolysaccharides were hydrolysed with 0·5 N-HCl in sealed tubes in boiling water complete liberation of hexosamines and reducing sugars occurred in about 2 hr. There was no detectable SO_4^{2-} ions either in the hydrolysates of the mucopolysaccharides or in the hydrolysate of the mucoprotein, which showed that sulphuric acid esters were absent from all three substances.

The hexosamines of the hydrolysed mucopolysaccharides and mucoprotein were allowed to

react for 20 min. at 100° with acetylacetone in a solution of the following composition: Na_2CO_3, 0·25 M; $NaHCO_3$, 0·1 M; NaCl, 0·1 M. After cooling, the resulting complexes were coupled with *p*-dimethylaminobenzaldehyde in 0·5 N-HCl containing 56 % (v/v) of ethanol, and further ethanol was added to give 65 % (v/v) ethanol, the final concentration of acid being 0·4 N-HCl. The solutions were allowed to stand at 22·5° for 24 hr. so that the complexes attained their stable absorption spectra. Fig. 2 gives the absorption-spectrum curve of the complex from the phenol-soluble mucopolysaccharide. Similar curves were obtained with the phenol-insoluble mucopolysaccharide and the mucoprotein. Also given in Fig. 2 are the absorption-spectrum curves of the corresponding complexes obtained after treating glucosamine and chondrosamine with acetylacetone and coupling with *p*-dimethylaminobenzaldehyde. It may be seen that the absorption maximum of the complex from the phenol-soluble mucopolysaccharide lies between that of the glucosamine compound and that of the chondrosamine compound. The absorption curve of the complex produced by treating an equal mixture (w/w) of glucosamine and chondrosamine with acetylacetone and coupling with *p*-dimethylaminobenzaldehyde was found to have an absorption maximum at 523 mμ. Thus it was assumed that the difference between the absorption spectra of the complexes from hydrolysates of the mucopolysaccharides and the mucoprotein and that of the complex from glucosamine was due to chondrosamine, the probable presence of which was also indicated, with the mucopolysaccharides, by chromatography.

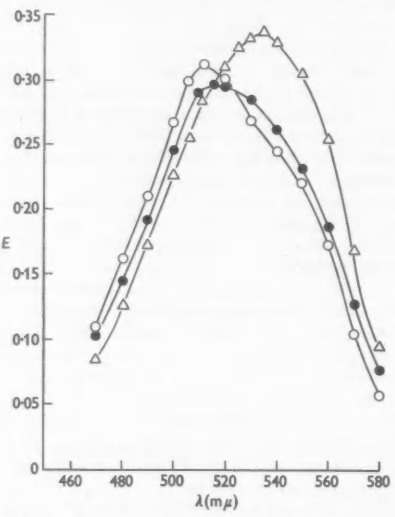

Fig. 2. Absorption-spectrum curve of the complex obtained by treating the hexosamines from the hydrolysate of the phenol-soluble mucopolysaccharide with acetylacetone and coupling with *p*-dimethylaminobenzaldehyde, compared with the absorption-spectra curves of the corresponding complexes obtained from glucosamine and chondrosamine. The $\lambda_{max.}$ for the complex from the phenol-soluble mucopolysaccharide hydrolysate (●) was 516 mμ, compared with the $\lambda_{max.}$ of the glucosamine complex (○), which was 512 mμ, and that of the chondrosamine complex (△), which was 535 mμ.

The sugars liberated by hydrolysis of the mucopolysaccharides and the mucoprotein with 0·5 N-HCl were identified by filter-paper partition chromatography. Ascending chromatograms were run at 22·5° in four solvent systems and the hydrolysates were irrigated on the same papers as the controls: glucosamine, chondrosamine, galactose, mannose and fucose and with mixtures of these reference sugars. With the phenol-insoluble and the phenol-soluble mucopolysaccharides, the collidine–water system resolved the hydrolysates into four components: (1) R_F 0·19, corresponding to chondrosamine; (2) R_F 0·23, to glucosamine; (3) R_F 0·33, to galactose; (4) R_F 0·48, to fucose. Similar results were obtained with the pyridine–butanol–benzene–water system and the phenol–water system, except that neither of these solvent mixtures separated the two amino sugars. The butanol–acetic acid–water system was able only to separate fucose from the other sugars. The mucoprotein hydrolysate was resolved by the pyridine–butanol–benzene–water system into four components corresponding to the amino sugars (R_F 0·23), galactose (R_F 0·28), mannose (R_F 0·35) and fucose (R_F 0·46). The results obtained with the other three solvent systems confirmed the presence of these sugars in the hydrolysate, but no solvent system resolved the amino sugars, despite spectrophotometric evidence that was obtained of the presence of both glucosamine and chondrosamine. It was concluded therefore that the hydrolysates of the mucopolysaccharides contained glucosamine, chondrosamine, galactose and fucose, and that the hydrolysate of the mucoprotein contained glucosamine and possibly chondrosamine, galactose, mannose and fucose.

The amino acids liberated from the phenol-insoluble and phenol-soluble mucopolysaccharides by hydrolysis with 6 N-HCl were identified by two-dimensional paper partition chromatography. Evidence was obtained of the presence of the following amino acids in the mucopolysaccharides: arginine, histidine, lysine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic acid, glycine, alanine and proline. Spots corresponding to cystine, tyrosine and phenylalanine were also

obtained, but these were not as clearly defined. Two other ninhydrin-reacting areas were observed in the chromatograms of both mucopolysaccharide hydrolysates, but these were never seen in chromatograms of mixtures of the series of amino acids listed above. The larger area was reproduced when amino acid mixtures containing hexosamines were chromatographed under the same conditions. The smaller area, R_F 0·05 in the butanol–acetic acid–water system and R_F 0·08 in the pyridine–butanol–benzene–water system, could not be identified.

Comparison of the properties of the mucoproteins of sputum and of urine

As the mucoprotein from sputum had a lower polysaccharide content than that of the sputum mucopolysaccharides and, unlike them, contained mannose, it appeared to resemble the mucoprotein isolated by Tamm & Horsfall (1952) from urine, rather than either of the mucopolysaccharides. This urinary mucoprotein was therefore prepared so that its properties could be compared directly with those of the mucoprotein from sputum.

Both complexes were obtained as voluminous white powders which gave neutral aqueous solutions. Both were precipitated when the pH of these aqueous solutions was lowered below 3·3. Electrophoretic examination showed that each complex was essentially homogeneous. On hydrolysis, both mucoproteins were found to contain amino sugars, and galactose, mannose and fucose. Both Gottschalk (1952) and Odin (1952) have found these sugars to be components of the carbohydrate residue of the urinary mucoprotein. Similar patterns were obtained when hydrolysates of the mucoproteins were irrigated with the pyridine–butanol–benzene–water mixture on the same ascending-paper chromatogram, though the fucose spot of the sputum-mucoprotein hydrolysate was more intense than the corresponding fucose spot of the urinary-mucoprotein hydrolysate.

The two complexes, however, differed in the following ways. The sputum mucoprotein was soluble in 0·58 M-NaCl, whereas the urinary mucoprotein was precipitated completely from its aqueous solutions by 0·58 M-NaCl. The viscosities of comparable solutions (Table 2) indicated that the urinary mucoprotein was probably a larger molecule than the sputum mucoprotein. The measurements were carried out in salt-free aqueous solutions of the two complexes because the presence of even low concentrations of inorganic salts may have caused the urinary mucoprotein to assume a heterogeneous disperse phase and thus to give rise to false viscosity readings. The relative viscosity of the sputum mucoprotein in water was much higher than the relative viscosity of the same complex in aq. 0·85% NaCl soln. This difference may be due to the electroviscous effect described by Bull (1940).

The ultraviolet-absorption spectra of the two complexes also differed (Fig. 3), the sputum mucoprotein showing a peak at 259 mμ and the urinary mucoprotein a peak at 278 mμ. In Table 2 it may

Table 2. *Comparison of the composition and properties of the mucoproteins of sputum and of urine*

Analyses of the mucoproteins were carried out on samples which were dried at 56° for 1 hr. The analytical results are expressed as a percentage of this dry weight. For methods of analysis, see text.

	Sputum mucoprotein	Urinary mucoprotein
Total N (%)	8·7	10·3
Amino sugars (%)	14·3	8·4
Reducing sugars (%)	26·3	16·7
Total P (%)	0·75	0·05
Inorganic P (%)	0·01	Nil
Organic P (%)	0·74	0·05
Relative viscosity (ν)*	5·31	5·81

* Viscosity measurements were made on 0·2% solutions of the mucoproteins in water.

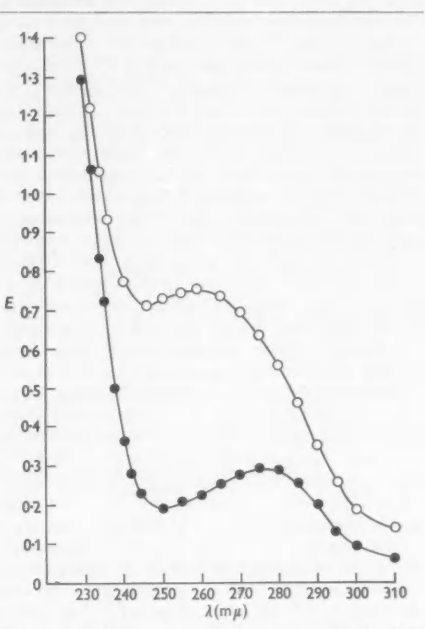

Fig. 3. Ultraviolet-absorption-spectra curves of 0·025% solutions of the mucoproteins of sputum (○) and urine (●) in 0·025M-Na_2HPO_4–NaH_2PO_4 buffer, pH 6·8. The $\lambda_{max.}$ for the sputum mucoprotein was 259 mμ and the $\lambda_{max.}$ for the urinary mucoprotein was 275 mμ.

be seen that this difference is reflected in the organic phosphorus contents of the two complexes and was possibly due to the presence of nucleic acids in the sputum mucoprotein. The analytical figures in Table 2 show that the sputum mucoprotein had a smaller total nitrogen content and a greater hexosamine and reducing-sugar content than the urinary mucoprotein had. The sputum mucoprotein was therefore a complex which contained approximately 50 % of polysaccharide, in contrast with the urinary mucoprotein which contained approximately 40 % of polysaccharide. The sputum mucoprotein was also shown to contain alkali-labile *N*-acetylhexosamine linkages (Morgan & Elson, 1934), whereas it was not possible to demonstrate the existence of such linkages in the urinary mucoprotein.

DISCUSSION

The method of isolating the mucopolysaccharides and the mucoprotein is suitable only for revealing the nature of the carbohydrate constituents of sputum. The total yield of material after peptic digestion, estimated on a weight for weight basis with acetone-dried sputum, was less than 40 %, indicating that these carbohydrate constituents probably make up less than half of the total solids present in pooled sputum. This approximate estimate is also in accord with that obtained from the analysis of acetone-dried sputum, the remainder of the high-molecular-weight components being either proteins or nucleoproteins. The enzymic digestion made it impossible also to obtain any information about the mechanism of coagulum formation. The method may, however, be considered adequate for isolating carbohydrate constituents, as it has been used to isolate blood-group mucopolysaccharides from saliva and from human stomachs (Kabat *et al.* 1947), the specific activities of these mucopolysaccharides being retained to a high titre. Similarly, the phenol-fractionation technique of Morgan & King (1943), which was used to purify the mucopolysaccharides, has been employed by Aminoff, Morgan & Watkins (1950) and Annison & Morgan (1952*a*, *b*) to purify blood-group mucopolysaccharides from ovarian-cyst mucin; their work has shown that the method has no deleterious effect on the specific activity of the complexes. Thus although the final products isolated by such procedures from sputum probably cannot be equated with the carbohydrates in their native state in the lower respiratory tract, they were a valuable guide for assessing the type of carbohydrate complex present.

Analysis showed that the two mucopolysaccharide preparations from sputum are very similar in composition to the various blood-group mucopolysaccharides which have been isolated from human stomachs by Kabat *et al.* (1947), from meconium by Buchanan & Rapoport (1951) and from ovarian-cyst fluids by Aminoff *et al.* (1950) and Annison & Morgan (1952*a*, *b*). The component sugars of the carbohydrate moieties of the sputum mucopolysaccharides are identical with those of the blood-group mucopolysaccharides (Aminoff *et al.* 1950; Bendich, Kabat & Bezer, 1947). The sputum mucopolysaccharides possess alkali-labile *N*-acetylhexosamine linkages that are similar to those of the blood-group mucopolysaccharides (Aminoff *et al.* 1952). Furthermore, the amino acids of the polypeptide moiety of the sputum mucopolysaccharides are closely similar to those of the polypeptide moiety of the blood-group mucopolysaccharides (Aminoff *et al.* 1950; Annison & Morgan, 1952*a*, *b*). The finding of several molecular species of mucopolysaccharide in the pooled sputum obtained from a large number of patients also suggests that these complexes belong to the same class of substance as blood-group mucopolysaccharides, since considerable differences in molecular dimensions are found between type-specific mucopolysaccharides from different blood groups (Kekwick, 1952*a*, *b*).

In contrast with the several molecular species of mucopolysaccharide that were isolated from pooled sputum, only one essentially homogeneous mucoprotein was obtained. This complex is distinct in composition from the mucopolysaccharides and possesses different physical and chemical properties. It bears more resemblance to the mucoprotein that can be isolated from urine by the method of Tamm & Horsfall (1952) than to either of the sputum mucopolysaccharides. The polysaccharide moiety of the sputum mucoprotein is composed of the same sugars as that of the urinary mucoprotein, but the urinary mucoprotein does not possess the alkali-labile *N*-acetylhexosamine linkages that are present in the sputum mucoprotein. Analysis showed that the urinary mucoprotein has a larger polypeptide content than the sputum mucoprotein, and viscosity measurements indicated that the urinary mucoprotein is probably the larger molecule of the two. The sputum mucoprotein possibly contains nucleic acids which apparently form part of its molecular structure.

Some of these differences between the mucoprotein of sputum and that of urine may have been due to the manner of preparing the sputum mucoprotein, which involved enzymic digestion of acetone-dried sputum. This material contained nucleoprotein debris from the leucocytes and epithelial cells which are present, particularly in mucopurulent sputum. Not only may the enzymic digestion have had the possible effect of degrading the mucoprotein complex, but it could also have

released nucleic acids from denatured nucleoproteins of cellular origin which in turn, by election, may have formed a complex with the mucoprotein. The sputum mucoprotein in the native state therefore may resemble more closely the urinary mucoprotein so far as it may be of a molecular size more comparable with that of the complex from urine; in addition, it may not contain nucleic acids as part of its molecular architecture.

The question may be raised whether the mucopolysaccharides and the mucoprotein of sputum are present solely as an outcome of morbid change. Similar polysaccharides are produced at other sites by apparently normal epithelial surfaces which possess either goblet cells or the tubuloalveolar type of muco-serous glands, or both. These carbohydrate complexes which occur in sputum may therefore, either wholly or partly, be regarded as products of the secretory or hypersecretory activities of the bronchial epithelium, possibly modified by pathological changes.

SUMMARY

1. A method has been described for isolating two mucopolysaccharide fractions and an electrophoretically homogeneous mucoprotein from pooled acetone-dried sputum.

2. The two mucopolysaccharides, one insoluble and the other soluble in 90% (w/v) phenol–water mixture, contained 5·2 and 5·7% of nitrogen respectively; they were similar in composition to the blood-group mucopolysaccharides.

3. The mucoprotein, which contained 8·7% of nitrogen, was found to be distinct from either of the mucopolysaccharides isolated from sputum, but to have some properties in common with the mucoprotein that can be isolated from urine.

4. The polysaccharide moieties of the mucopolysaccharides were composed of *N*-acetylglucosamine, *N*-acetylchondrosamine, galactose and fucose; the mucoprotein contained mannose in addition to these sugars.

5. The phenol-insoluble mucopolysaccharide was electrophoretically non-homogeneous, showing two components which moved at similar velocities. In contrast, the sputum mucoprotein was essentially homogeneous.

6. These carbohydrate complexes constitute less than 50% of the total solids of pooled sputum. They represent, either wholly or partly, the secretory activities of the bronchial epithelium possibly modified by disease; they may have suffered some degree of degradation during their isolation.

The author is indebted to Dr F. Himmelweit for both his introduction to this work and his continued interest in its progress. Grateful acknowledgements are further due to Professor W. T. J. Morgan and Professor A. Neuberger for valuable advice and suggestions.

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Acidic Peptides of the Lens

5. *S*-SULPHOGLUTATHIONE*

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The occurrence in lens extracts of acidic peptides, apparently derived from glutathione, was described in the first paper of this series (Waley, 1956). These compounds were isolated by electrophoresis; they migrated rapidly towards the anode at pH 4 (the 'fast-acid' fraction). A major constituent of this fraction (called 1α4) was shown to have glutamic acid as the *N*-terminal amino acid of a tripeptide, which gave also cystine and glycine on hydrolysis. This paper describes further work leading to the identification of this derivative as the thiosulphate related to glutathionine; following the suggestion of Swan (1957), the thiosulphate will be called *S*-sulphoglutathione. The main analytical technique employed has been ion-exchange chromatography; *S*-sulphoglutathione is the most acidic ninhydrin-positive compound in lens extracts and can be readily isolated by using a buffer of high ionic strength so that all the other compounds are barely retarded.

EXPERIMENTAL

Analytical techniques

Separation of peptides from proteins. Previously (Waley, 1956), dialysis in alkaline solution was used to remove high-molecular-weight compounds; the pH was maintained at a high value so as to minimize enzymic reactions. Such conditions, however, are likely to lead to oxidation. Therefore two other methods of removing proteins have also been used. One is heat-precipitation at pH 5·3 (Hunter, 1957) and the other is precipitation with trichloroacetic acid.

Fractionation of acidic compounds. Anion-exchange chromatography has been used to separate these strongly acidic compounds. As the earlier work had shown that the 'fast-acid' fraction contained nucleotides, chromatographic methods were similar to those developed for nucleotides (Hurlbert, Schmitz, Brumm & Potter, 1954).

In these methods an anion-exchange resin (Dowex 1 formate) is used, and the acidic species are eluted by a gradually increasing concentration of formic acid or ammonium formate (gradient elution, Alm, Williams & Tiselius, 1952). It is inconvenient to use ammonium formate as the eluent for ninhydrin-positive compounds, but the formic acid system proved quite suitable and was used in one of the experiments described below. As an example of the resolving power of this system, it may be mentioned that the sulphonic acid (GSO_3H) was well separated from *S*-sulphoglutathione ($GSSO_3H$), although these two compounds have similar electrophoretic mobilities. There is, however, one disadvantage of the formic acid system: the pH of each of the fractions has to be individually adjusted before carrying out the ninhydrin reaction. The use of a pyridine formate buffer (described below) overcomes this disadvantage: this system is well suited for the isolation of *S*-sulphoglutathione formed enzymically from *S*-sulphocysteine; moreover, pyridine formate buffers, being more volatile than ammonium formate ones, can be removed by addition of water and evaporation at the pressure attained by a water pump. The separation of a synthetic mixture of *S*-sulphoglutathione and *S*-sulphocysteine is shown in Fig. 1.

Materials

S-Sulphoglutathione. This compound was prepared by the reaction of oxidized glutathione (GSSG) with excess of Na_2SO_3 (Clarke, 1932). Auto-oxidation of glutathione (GSH) to the disulphide was accomplished by exposing it (1·23 g.) in 0·4 N-NaOH (10 ml.) containing $CHCl_3$ (to

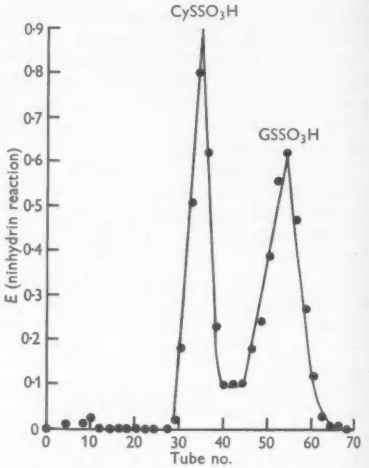

Fig. 1. Chromatography of a mixture of synthetic *S*-sulphoglutathione (7·9 μmoles) and *S*-sulphocysteine (8·8 μmoles) on Dowex 1 formate column (28 cm. × 1·2 cm.). Elution was with M-pyridine formate, pH 5·4; the eluate was collected in 3 ml. fractions.

* Part 4: Cliffe & Waley (1958).

prevent bacterial growth) and a trace of $FeSO_4$, to the air for 48 hr.; as the oxidation proceeds, the red colour fades. The disulphide thus obtained gave only one ninhydrin-positive spot on paper chromatography in the solvent described below. The use of hydrogen peroxide (Pirie, 1931; Schöberl & Hornung, 1938) tends to give material contaminated with a little of the sulphonic acid (GSO_3H).

The reaction of GSSG with excess of Na_2SO_3 was carried out under conditions such that the GSH first formed would be re-oxidized to GSSG. This facilitates isolation of the product. The pH must be kept close to pH 7, as at higher pH values the equilibrium is unfavourable (Cecil & McPhee, 1955), and at lower ones the reaction (and auto-oxidation) is too slow.

The solution of GSSG obtained above was treated with $Na_2SO_3,7H_2O$ (5 g.), and acetic acid added to lower the pH to 6·9 and O_2 bubbled through for 24 hr. Further Na_2SO_3, $7H_2O$ (5 g.) was added and the passage of O_2 continued for 48 hr. The solution was treated with a cation-exchange resin [Amberlite IR-120 (H), British Drug Houses Ltd.] (7 g.) and freed from SO_2 by passing N_2 through. Barium acetate (25%, w/v; 20 ml.) was added, and barium sulphate centrifuged down and ethanol (75 ml.) added to the supernatant. The *barium salt* of *S-sulphoglutathione* was collected and dried in air (yield 0·88 g., 37%); $[\alpha]_D^{19}+44°$ (*c*, 1·3 in water) (Found: C, 20·1; H, 3·8; N, 6·9; S, 10·5; Ba, 22·7. $C_{10}H_{15}O_9N_3S_2Ba,4H_2O$ requires C, 20·2; H, 3·9; N, 7·1; S, 10·8; Ba, 23·1%).

The absence of GSSG from the product can be checked by paper chromatography, or by paper electrophoresis at pH 4. The paper chromatography was carried out with butanol–acetic acid–water–pyridine (30:6:24:20, by vol.) as solvent on no. 4 paper; in a 16 hr. run, GSSG moves about 3 cm. and $GSSO_3H$ about 9 cm. The solvent runs off the end of the paper, so R_F values are not quoted, but an available compound which travels the same distance as $GSSO_3H$ is aspartic acid.

Earlier preparations of $GSSO_3H$ were contaminated with GSSG and, on occasions, with GSO_3H. Ion-exchange chromatography, under the conditions described below ('heat-precipitation experiment') was used to isolate pure $GSSO_3H$ from these mixtures.

RESULTS

Alkaline-dialysis experiment

Calf lenses (30 g.) were added to cooled triethylamine formate buffer (prepared from 12·5 ml. of triethylamine, 2·25 g. of 90% formic acid and 90 ml. of water), and macerated and dialysed as described previously (Waley, 1956; experiment L. II). The diffusate was concentrated to a syrup, and extracted with $CHCl_3$ and dried *in vacuo*. The residue was dissolved in water (25 ml.) and fractionated on a column 1·2 cm. × 28 cm. long of Dowex 1 formate. The resin (Dow Chemical Co., Michigan; 4% cross-linked, 200–400 mesh, 440 g., moist chloride form) had been converted into the formate form and purified as follows. It was washed with water by decantation until the supernatant was clear, and then transferred to a Büchner funnel and washed with 3 M-sodium formate (4 l.), and then made into a column in a separating funnel and washed with 90% formic acid (1 l.). Finally the resin was washed free from acid with water. A portion (about 30 ml.) of the resin was added to 60 ml. of M-pyridine formate buffer, pH 5·4 [42 ml. of 90% formic acid and 162 ml. of purified pyridine and water to 1 l.; the pyridine was purified by distillation under N_2 after adding Br_2 (2 ml./l.)], and de-gassed at the water pump and the column packed; the buffer used for elution was also de-gassed.

The solution of the extract was brought to pH 5 with N-NaOH and then added to the column during 3 hr. The column was eluted overnight with the M-pyridine formate buffer at a rate of 12 ml./hr.; fractions of 3 ml. were collected. Portions (1 ml.) of the even-numbered tubes were taken for ninhydrin estimation; the modification of Rosen (1957) of the method of Yemm & Cocking (1955) was used. The high concentration of formate in the buffer used for elution prevents the adsorption of any but the most strongly acidic species; thus glutamic acid, glutathione and ophthalmic acid are not retained by the column and form part of the large peak in Fig. 2. The contents of two sets of tubes, 31–39 and 48–58, were concentrated, and water added and removed by distillation several times to remove most of the buffer. The materials were then examined by paper chromatography in butanol–acetic acid–water–pyridine and by paper electrophoresis at pH 4 (Fig. 3; the apparatus of Cliffe & Waley, 1958, was used).

The second fraction (tubes 48–58) showed only one ninhydrin-positive spot, which travelled at the same rate as authentic $GSSO_3H$ in paper chromatography and paper electrophoresis; comparison of

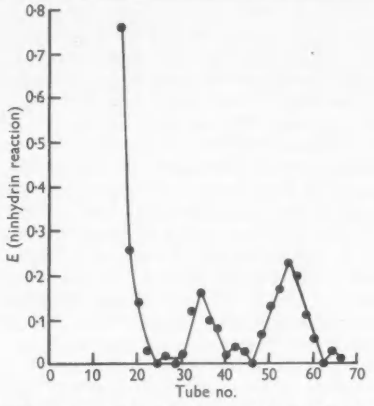

Fig. 2. Chromatography of diffusate obtained by dialysis of an extract of calf lenses (30 g.). Chromatography was carried out as described for Fig. 1.

Figs. 1 and 2 shows that this substance also emerged from the ion-exchange column at the position expected for $GSSO_3H$. The amount of this substance (assuming it to be $GSSO_3H$), calculated from the area of the peak in Fig. 2, was 2·5 μmoles; this corresponds to a concentration of 10 μmoles/100 g. of calf lens.

The ninhydrin-positive material in tubes 31–39 seems to be labile, as after evaporation only weak and ill-defined spots were observed when the residue was examined by paper electrophoresis.

Heat-precipitation experiment

Thirty calf lenses were macerated with water (120 ml.); 1% acetic acid (24 ml.) was added to lower the pH to 5·1, and the mixture was poured into centrifuge tubes, and heated with stirring at 90° for 4 min., and cooled and the protein centrifuged down. The supernatant was treated with Hyflo Supercel (Johns-Manville) and filtered. The precipitate was extracted with 0·03% acetic acid (120 ml.), and the extract clarified as before. The extracts were passed through a column (1·2 cm. × 15 cm. long) of Dowex 1 formate (10% cross-linked), equilibrated with the pyridine formate buffer diluted 1:10 (pH 5·5). The elution was then carried out with a linear gradient between the 0·1 M-pyridine formate (110 ml.) in the mixing vessel, and 4 M-formic acid (110 ml.) in the reservoir. The two vessels (volume 1 l.) were connected (at the bottom) by a short length of capillary tubing, and the mixing vessel was stirred by a magnetic stirrer. Titration against standard alkali confirmed that the concentration of formic acid in the eluate increased approximately linearly. The eluate was collected in 2 ml. fractions; after 77 fractions, the mixing vessel was replenished with 3 M-formic acid (100 ml.) and the reservoir with 4 M-formic acid (100 ml.). The tubes were analysed by spotting on paper and developing with ninhydrin; after the first 20 tubes, there was ninhydrin-positive material in tubes 105–125; the peak at tube 118 was close to that previously obtained with authentic $GSSO_3H$. The contents of tubes 105–125 were concentrated, and the presence of material which was chromatographically homogeneous and chromatographically indistinguishable from $GSSO_3H$ was confirmed.

The bulk of the material in water (0·1 ml.) containing barium acetate (0·7 mg.) was diluted with ethanol (0·3 ml.), and the barium salt centrifuged down and used in the comparisons of the reactions of the material from the lens with those of authentic $GSSO_3H$ (see below).

The amount of $GSSO_3H$ obtained in this experiment was not measured, but it was of the same order as that obtained in the preceding experiment; in a repetition of the heat-precipitation experiment, however, much less $GSSO_3H$ was isolated.

Trichloroacetic acid experiment

In this experiment the bulk of the lens proteins were removed by precipitation with cold 8% (w/v) trichloroacetic acid, and the supernatant was extracted with ether, and brought to pH 6 and freed from residual protein by ultrafiltration under pressure through a dialysis sac. The fractionation was essentially similar to that described in the heat-precipitation experiment. *S*-Sulphoglutathione was isolated, in amount corresponding to approx. 3 μmoles/100 g. of calf lens.

In another experiment of this type, similar except that the ultrafiltration step was omitted, no $GSSO_3H$ could be detected. The variations in the amount of $GSSO_3H$ isolated are discussed below.

Enzymic synthesis of S-sulphoglutathione

Dialysed calf-lens extracts were used as a source of enzymes, and *S*-sulphocysteine (Clarke, 1932), glutamic acid and glycine as substrates, under the conditions previously described (Cliffe & Waley, 1958). I am indebted to Mr E. E. Cliffe for carrying out the incubation. The incubation mixture contained adenosine triphosphate (ATP) (2·5 mM), 3-phosphoglyceric acid (22 mM), KCl (42 mM), $MgSO_4$ (10 mM), potassium phosphate buffer, pH 6·4 (6 mM), L-glutamic acid (8 mM), *S*-sulphocysteine (8 mM) and glycine (8 mM). The total volume of the solution was 20 ml. and it contained 15 ml. of dialysed calf-lens preparation. The reaction was carried out at pH 6·5 at 37° for 24 hr.; a portion (15 ml.) of the solution was treated with hot ethanol (38 ml.) and the protein centrifuged down. The supernatant was concentrated to 2 ml., and adsorbed on a 1·2 cm. × 28 cm. long column of Dowex 1 formate (4% cross-linked) equilibrated with M-pyridine formate and eluted with this buffer overnight, fractions (2·8 ml.) being collected every 15 min. The tubes in the position of *S*-sulphoglutathione were ninhydrin-positive, and concentration of the material in these tubes and analysis by paper chromatography and paper electrophoresis confirmed that *S*-sulphoglutathione had been formed by the enzymic reaction.

Reaction of S-sulphoglutathione with iodine

Iodine (1·1 mg.) in ethanol (0·5 ml.) was added to the barium salt of *S*-sulphoglutathione (4·7 mg.) in water (0·5 ml.) and the mixture kept at 100° for 10 min. Analysis of the reaction was by paper electrophoresis at pH 4 and showed that the usual conversion of an alkyl thiosulphate into the disulphide (here GSSG) took place. The conversion was incomplete; addition of more iodine and further heating for 5 min. at 100° caused further oxidation of the GSSG to GSO_3H. When a specimen (about 0·2 mg.) of the barium salt obtained from

calf lens (heat-precipitation experiment) in water (0·02 ml.) was heated with iodine (0·04 mg.) in ethanol (0·02 ml.) for 10 min. at 100°, GSSG was again formed.

Reaction of S-sulphoglutathione with cysteine

Cysteine hydrochloride hydrate (2·9 mg.) in 0·05N-NaOH (0·5 ml.) was added to the barium salt of *S*-sulphoglutathione (4·5 mg.) in water (0·5 ml.); a similar experiment (on a smaller scale) was carried out with the barium salt from the lens. The reaction mixtures were analysed by paper electrophoresis at pH 4 (Fig. 3). Both samples of barium salts gave a product whose mobility is consistent with its formulation as the mixed disulphide (CyS-SG) from cysteine (CySH) and GSH. In addition, spots in the positions of GSH and GSSG can be seen; these probably arise from the decomposition of the mixed disulphide (Swan, 1957).

Decomposition of S-sulphoglutathione in lens extracts

Several experiments were carried out to see if *S*-sulphoglutathione, added to lens extracts, was stable or not. Two kinds of extract were used. In the first, calf lenses were ground with their own weight of water, and the dispersion (referred to as 1:1 extract) was used directly, or diluted; in the second this dispersion was dialysed for 48 hr. and centrifuged. In each case the reaction mixture, consisting of 0·055M-barium salt of $GSSO_3H$ (20 μl.) and the lens extract (60 μl.), was kept at room temperature for 50 min. The pH was then brought to 5, and the protein removed by centrifuging after heat denaturation (100° for 4 min.). Portions (5 μl.) of the supernatant were examined by paper electrophoresis at pH 4 to see if the $GSSO_3H$ was still present.

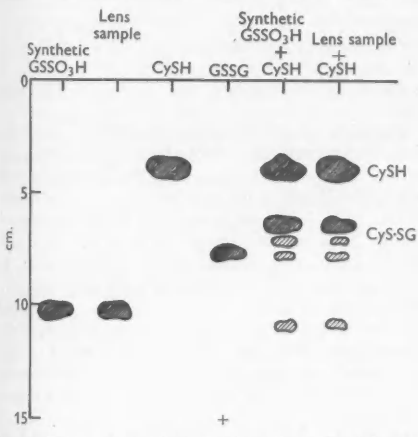

Fig. 3. Paper electrophoresis of synthetic *S*-sulphoglutathione, the lens specimen and the products of their reaction with cysteine. Electrophoresis was at pH 4 on no. 52 paper for 4 hr. at 8 v/cm.

S-Sulphoglutathione was stable in the dialysed lens extract, and in a diluted lens extract (the 1:1 extract diluted ten times). But when the 1:1 extract was neither diluted nor dialysed the *S*-sulphoglutathione decomposed; this result was obtained repeatedly, but not invariably.

On some occasions the *S*-sulphoglutathione was stable. The variable recovery of $GSSO_3H$ added to lens extracts may be the cause of the variability in the amount of $GSSO_3H$ isolated from lens extracts.

DISCUSSION

Evidence for the presence of S-sulphoglutathione in lens extracts

That the strongly acidic peptide ('1α4') previously encountered in lens extracts was closely related to glutathione was suggested by hydrolysis to the amino acids of glutathione (Waley, 1956). The strongly acidic group was, however, unidentified. It has now been shown that this compound does not contain phosphorus, so the possibility that the compound was a nucleotide peptide may be discounted. The strongly acidic group was therefore probably a sulphonic acid, but its location in the glutathione must be such that the only amino acids liberated on hydrolysis are those derived from glutathione. The only simple way of accommodating these facts is by replacing the thiol hydrogen of GSH by the sulphonic acid (SO_3H) radical, so that the compound is the thiosulphate, $GSSO_3H$; the thiosulphate group in these compounds is known to be unstable to acid hydrolysis.

Clarke (1932) described the preparation of a compound believed to be this thiosulphate, although it could not be purified; he used the reaction:

$$GSSG + SO_3^{2-} + H^+ \rightleftarrows GSSO_3^- + GSH \qquad (1)$$

It has now been found that the *S*-sulphoglutathione can be characterized as a barium salt; in earlier experiments, the thiosulphate could be purified by ion-exchange chromatography to separate it from GSH or GSSG, but if oxygen is passed through the reaction mixture the GSH formed is re-oxidized and reacts further.

This synthetic sample of *S*-sulphoglutathione was compared with the material obtained from the lens extracts. There were no differences on paper chromatography, paper electrophoresis and ion-exchange chromatography. A direct comparison of X-ray-powder photographs was impossible as the barium salts are not sufficiently crystalline to give

a characteristic diagram. Fortunately, however, thiosulphates undergo several characteristic reactions which confirm the nature of the material from the lens. Thus heating a solution of a thiosulphate with iodine gives the disulphide, a reaction often used preparatively. Another reaction (which proceeds rapidly at room temperature in neutral solutions) is the reaction with thiols (Swan, 1957; Schöberl & Bauer, 1957), and *S*-sulphoglutathione reacted with excess of cysteine (Fig. 3) to give a compound which is presumably the mixed disulphide formed by the following reaction:

$$GSSO_3^- + CySH \rightarrow GSSCy + SO_3^{2-} + H^+ \quad (2)$$

In both these reactions, the material in the lens extracts behaved in the same way as the synthetic *S*-sulphoglutathione. It is therefore concluded that the lens extracts do contain *S*-sulphoglutathione, and it will now be considered whether the substance is formed in the course of the extraction and fractionation.

Is S-*sulphoglutathione an artifact?*

It is an obvious possibility that *S*-sulphoglutathione might be formed in some way from glutathione; there is only about one-hundredth as much of the thiosulphate as there is of glutathione in the lens extract. *S*-Sulphoglutathione was first isolated from extracts which had, during the dialysis, been exposed to a high pH value for several days. In subsequent experiments, however, it was isolated from extracts from which the proteins had been removed by precipitation with trichloroacetic acid, or by heating for a short time at pH 5. These conditions are clearly less likely to lead to oxidative changes. Moreover, if the thiosulphate is being formed from glutathione during the course of isolation, the only known reaction by which this could happen would be the reaction of sulphite with GSSG (reaction 1). As the apparent equilibrium constant of reaction (1) is of the order of unity at pH 7, concentrations of glutathione, *S*-sulphoglutathione and sulphite at equilibrium could be 10, 0·1 and 1 mM respectively (the concentration of glutathione in calf lens is about 10 mM and the concentration of *S*-sulphoglutathione is about 0·1 mM). It is not known whether the lens contains sulphite, but even if a value for the concentration of sulphite were available, it would not indicate whether the sulphite was a cause of the presence of *S*-sulphoglutathione (reaction 1 proceeding to the right) or a consequence of it (reaction 1 proceeding to the left). Moreover, glutathione reductase is present in the lens (van Heyningen & Pirie, 1953); in the presence of reduced triphosphopyridine nucleotide (TPNH), this enzyme catalyses the apparently irreversible reduction of GSSG. Thus this system will displace the equilibrium of reaction (1) to the left, and is probably the cause of the decomposition of $GSSO_3H$ by lens extracts:

$$H^+ + TPNH + GSSG \rightarrow 2GSH + TPN^+$$

$$GSSO_3^- + GSH \rightarrow GSSG + SO_3^{2-} + H^+$$

Sum: $TPNH + GSSO_3^- \rightarrow GSH + SO_3^{2-} + TPN^+$ (3)

The rate of decomposition of $GSSO_3H$ will be affected by many factors, including the concentration of TPNH and the activity of glutathione reductase. It was observed above that $GSSO_3H$ was usually, but not invariably, decomposed by lens extracts, and it has been pointed out that lens extracts sometimes show no glutathione reductase activity (van Heyningen & Pirie, 1953); the isolation of *S*-sulphoglutathione from lens extracts was also sometimes unsuccessful. It seems possible that these observations are consequences of the reactions mentioned.

It should be noted that the products of reaction (3) as written above include sulphite and triphosphopyridine nucleotide (TPN^+). If these reacted according to reaction (4):

$$H_2O + SO_3^{2-} + TPN^+ \rightarrow TPNH + SO_4^{2-} + H^+ \quad (4)$$

then the overall reaction would be:

$$H_2O + GSSO_3^- \rightarrow GSH + SO_4^{2-} + H^+ \quad (5)$$

Whether reaction (4) takes place depends partly on how rapid alternative processes for the oxidation of sulphite are. It is particularly interesting that the oxidation of sulphite in mammalian tissues is accelerated by thioctic acid ($\overline{RSSR'}$) probably via the alkyl thiosulphate, $\overline{RSH\ R'SSO_3H}$, with hypoxanthine as cofactor, (Handler & Fridovich, 1956). This process may thus be formally similar to the one discussed above, except that GSSG is replaced by thioctic acid and TPN by hypoxanthine.

Although it has been assumed implicitly that the only thiol groups that $GSSO_3H$ will react with are those of glutathione, it is, of course, possible that $GSSO_3H$ will react with the thiol groups of proteins which, in the lens, outnumber the glutathione thiol groups. This reaction would give a mixed disulphide derived from the protein and glutathione. It is not known to what extent this reaction occurs.

Thus the situation is too complex to decide whether the *S*-sulphoglutathione in lens extracts arises from GSSG and sulphite by reaction (1); but it seems at least as likely that the concentration of $GSSO_3H$ in the cell is far greater than it is in the extracts.

It should also be considered whether the *S*-sulphoglutathione could arise by any other reactions, enzymic or otherwise. There are no other non-enzymic reactions known to convert GSH into

$GSSO_3H$. There is, however, a possible enzymic route to *S*-sulphoglutathione. Several analogues of glutathione are synthesized from their constituent amino acids by lens extracts (Cliffe & Waley, 1958), so the synthesis of $GSSO_3H$ from glutamic acid, *S*-sulphocysteine and glycine was investigated. It was, in fact, found that *S*-sulphoglutathione is built up; but unfortunately this answer is equivocal. The $GSSO_3H$ could be formed by direct reactions of the same type as lead to glutathione (omitting ATP):

$$\text{Glu} + \text{CySSO}_3\text{H} \rightarrow \gamma\text{-Glu}\cdot\text{CySSO}_3\text{H}$$

$$\gamma\text{-Glu}\cdot\text{CySSO}_3\text{H} + \text{Gly} \rightarrow \text{GSSO}_3\text{H}$$

but equally well the following indirect mechanism may hold:

$$\text{GSH} + \text{CySSO}_3^- \rightleftarrows \text{CySSG} + \text{SO}_3^{2-} + \text{H}^+$$

$$\text{CySSG} + \text{SO}_3^{2-} + \text{H}^+ \rightleftarrows \text{GSSO}_3^- + \text{CySH}$$

$$\text{Glu} + \text{CySH} + \text{Gly} \rightarrow \text{GSH}$$

$$\text{Sum: } \text{Glu} + \text{CySSO}_3^- + \text{Gly} \rightarrow \text{GSSO}_3^-$$

There is no evidence that *S*-sulphocysteine is present in the lens, so this enzymic reaction does not provide information about how the *S*-sulphoglutathione in lens extracts is formed.

Possible metabolic role of S-*sulphoglutathione*

If *S*-sulphoglutathione is present in lens cells, then the oxidation of TPNH to TPN^+ shown in reaction (3) could be important, especially in the carbohydrate metabolism of the lens. The suggestion that GSSG, by regenerating TPN, plays a part in the pentose phosphate cycle has been put forward by Kinoshita & Masurat (1957) and has been discussed recently elsewhere (Waley, 1958). The 'missing step' in the earlier scheme was the conversion of GSH into GSSG; if $GSSO_3H$ is present, then (by reaction 3) this difficulty is overcome; but then the 'missing step' (i.e. step for which there is no evidence about whether it occurs in the cell, and if so, how) becomes the formation of $GSSO_3H$. An enzymic mechanism for the formation of $GSSO_3H$ from precursors known to be present in the lens would go some way towards establishing this scheme.

SUMMARY

1. A derivative of glutathione has been isolated from calf-lens extracts.

2. This derivative has been identified as the thiosulphate ($GSSO_3H$) corresponding to glutathione (GSH); the identification is based on comparison of the physical and chemical properties of the material from the lens with those of synthetic $GSSO_3H$.

3. Lens extracts catalyse the synthesis of $GSSO_3H$ from glutamic acid, the thiosulphate ($CySSO_3H$) derived from cysteine and glycine.

4. The isolation of $GSSO_3H$ from lens extracts was accomplished by ion-exchange chromatography; elution was effected with pyridine formate buffers, which are volatile and do not react with ninhydrin.

I should like to thank my colleagues at this Laboratory for advice and encouragement, Mrs Little and Mrs Davies for the collection of calves' eyes, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation, the National Council to Combat Blindness, Inc., New York, for a 'Fight for Sight' grant-in-aid for chromatographic equipment and The Distillers Co. Ltd. for a generous gift of glutathione.

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A Specific Colorimetric Method for Estimation of Phlorrhizin in Biological Fluids

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The methods for estimation of phlorrhizin were reviewed by Lambrechts (1937), who pointed out that none of the methods discussed was specific. The most useful and sensitive, ultraviolet spectroscopy, does not distinguish between phlorrhizin and its aglucone phloretin. Furthermore, none of the methods was applicable to the estimation of phlorrhizin in urine. Braun, Whittaker & Lotspeich (1957) have described a method for estimation of phlorrhizin in urine, based on chromatographic separation combined with use of the Folin & Ciocalteu (1929) reagent. The method described here is based on the reaction used by Lehongre, Neumann & Lavollay (1950) for the estimation of hydrogen peroxide. Hydrogen peroxide in the presence of phlorrhizin gives a red colour, which is proportional to the amount of hydrogen peroxide present. We have found that if the amount of hydrogen peroxide is kept constant, the colour is proportional to the amount of phlorrhizin present. Neumann, Lehongre & Lavollay (1954, 1955, 1956*a*, *b*) claim that the preliminary step in the reaction is the formation of what they term 5-glucose-1:3:4:5-tetrahydroxybenzene by the Dakin (1909) reaction. This substance is then further oxidized in the presence of air to a red compound of unknown constitution.

METHODS

Phlorrhizin is first brought into solution in borate buffer (pH 9·1). The pH is important both because the rate of the reaction depends on pH and also because the final product acts as an indicator which is green at pH 12, red at pH 9 and yellow at pH 6·5. Since the rate of colour development also depends on electrolyte concentration the phlorrhizin is first extracted with butanol, and then re-extracted from the butanol with borate buffer. Hydrogen peroxide is then added to the borate extract and the colour compared with that of a standard phlorrhizin solution treated in a similar way.

Reagents. Hydrogen peroxide (100 vol.), 1 ml. diluted to 1 l., was used. Borate buffer (pH 9·1) was prepared by mixing 9 parts of 0·1 M-disodium tetraborate with 1 part of 0·4 M-boric acid. Barium hydroxide (0·3 N) and zinc sulphate (5%, w/v) solutions were prepared and standardized according to the directions of Somogyi (1945) for deproteinizing blood.

Preparation of phlorrhizin and its derivatives. Commercial phlorrhizin was purified by heating a solution (15 g./l.) to 80° for 10 min. with activated charcoal. The filtered solution was left overnight at 4°, and pure white crystals were obtained. Phloretin was prepared according to the method of Bach (1939), and recrystallized from acetic acid. Phlorin and phloretic acid were prepared by the method of Cremer & Seuffert (1912) and some phloretic acid was also prepared by the method of Neumann *et al.* (1956*a*). Phloroglucinol was obtained commercially.

Preparation of the standard. For estimation of phlorrhizin in clear solutions 0·2 mM-phlorrhizin in borate buffer (pH 9·1) can be used as standard. For biological fluids it is better to make up the standard in the appropriate fluid. By this means errors due to slight degrees of haemolysis of blood, presence of urinary pigments and losses in extraction are eliminated. These standards are prepared by addition of 0·4 ml. of aqueous 2 mM-phlorrhizin solution to 4 ml. of the fluid, e.g. bile or plasma. Preliminary trials showed that these standards gave results identical with those prepared by addition of solid phlorrhizin to the biological fluid. These standards were submitted to the same extraction procedure as the test solutions.

Extraction of phlorrhizin from biological fluids

Plasma. To 4 ml. of plasma were added 5 ml. of butanol and one drop of conc. HNO_3. The tube was shaken for 3 min. The emulsion formed was separated by centrifuging at 3000 rev./min. for 10 min. The butanol layer was pipetted off, and 3 ml. was added to 5 ml. of borate buffer. The mixture was shaken for 3 min. and then the aqueous layer was collected.

Bile. To decolorize the bile 1 ml. was added to 14 ml. of water, 2 ml. of 0·3 N-$Ba(OH)_2$ and 2 ml. of 5% $ZnSO_4$. The solution was shaken and filtered. To 5 ml. of filtrate in a separating funnel were added 5 ml. of butanol and one drop of conc. HNO_3. The butanol layer separated without centrifuging and the procedure was then continued as described for plasma.

Urine. To 5 ml. of urine in a separating funnel were added 5 ml. of butanol and one drop of conc. HNO_3, and the procedure was continued as described for bile.

Estimation of phlorrhizin

Into each of two test tubes was measured 2 ml. of borate extract of the standard, and into two other test tubes 2 ml. of borate extract of the unknown solution containing 0·02–2·0 mg. of phlorrhizin. To one tube of standard and one of unknown was added 1 ml. of hydrogen peroxide solution and to the other two tubes 1 ml. of water.

The tubes were left at room temperature for 2 hr., and the extinctions read at 490 mμ. The concentration of phlorrhizin in the unknown solution was calculated from the extinctions corrected by subtraction of those of the water blanks.

By ultraviolet spectroscopy. Phlorrhizin, according to Lambrechts (1937), may exist in either enol or keto form, the former with maximum extinction at 325 mμ, and the latter with maximum extinction at 285 mμ. In aqueous solution the form depends on pH, and in the method of Lambrechts (1937) for plasma the extinction is read at 325 mμ. In ethanolic solution phlorrhizin is present in the keto form. For estimation of phlorrhizin in dog bile, the bile is diluted 250–1000 times with ethanol; this is filtered and the extinction value is read at 285 mμ.

By chromatography. For urine, a modification of the chromatographic method of Braun *et al.* (1957) was used in which the final estimation of the eluate was carried out by u.v. spectroscopy instead of by the Folin & Ciocalteu reaction. Ascending chromatograms were run for 5 hr. at 25° in the non-aqueous phase of butanol–acetic acid–water (4:1:5, by vol.). Urine (up to 0·5 ml.) was spotted on paper from an Agla micrometer syringe. The spot should contain 5–500 μg. of phlorrhizin. If the spot contained more than 250 μg., the final position of phlorrhizin could be located by u.v. fluorescence. For smaller amounts a control spot from 0·1 ml. of ethanolic phlorrhizin solution (5 mg./ml.) was run. Squares of paper 3 cm. × 3 cm. containing the final spots were cut out and eluted with absolute ethanol. Elution by putting a filter paper into a solution of ethanol is unsatisfactory and is accompanied by considerable loss of phlorrhizin. We have found the most effective method is to suspend the square of paper and to run 5 ml. of absolute ethanol slowly over it from a dropping funnel. The extinctions of ethanol eluates were read at 285 mμ.

Spectroscopy. A Uvispek spectrophotometer with 1 cm. silica cuvettes was used.

RESULTS

Relation between phlorrhizin concentration and extinction. A series of solutions of phlorrhizin, from 0·02 to 2·0 mM, in borate buffer was made up. To 2 ml. of each solution without any preliminary extraction was added 1 ml. of hydrogen peroxide, and the extinction was measured after 2 hr. Over the range of concentrations tested the extinction varied in a linear manner with the original phlorrhizin concentration, an extinction of 1·0 corresponding with a concentration of 0·8 mM.

Effect of electrolyte concentration on colour development. In preliminary experiments it was observed that the use of two borate-buffer solutions of the same pH but different molar concentration leads to different rates of colour development. That electrolyte concentration can affect the rate of colour development is shown in Fig. 1. This source of error, which might be appreciable if solutions are concentrated before estimation, is prevented by preliminary extraction with butanol.

Estimation of phlorrhizin in plasma. Table 1 shows the results of estimating phlorrhizin in dog plasma. In these experiments phlorrhizin was added to plasma. The phlorrhizin was also estimated by the method of Lambrechts (1937). Plasma containing no phlorrhizin, when estimated by this method, always gives a blank value which varies in different plasma specimens, and if slight haemolysis has occurred the high blank values may interfere seriously with the estimation. With the hydrogen peroxide method even moderate amounts of haemolysis do not interfere with the estimation.

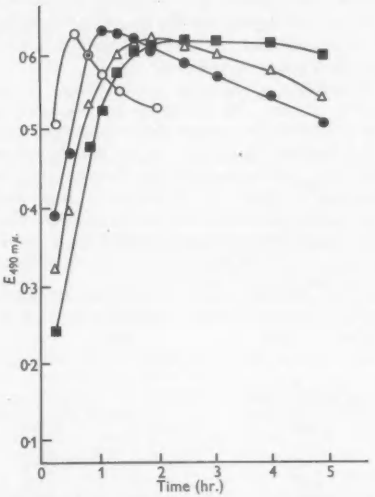

Fig. 1. Relation between the extinction value at 490 mμ and the time after adding the hydrogen peroxide. Hydrogen peroxide (1 ml.) was added to 2 ml. of 0·5 mM-phlorrhizin made up in borate buffer (■), borate buffer containing 0·5% of NaCl (△), borate buffer containing 10% (w/v) of NaCl (●) and borate buffer containing 20% (w/v) of NaCl (○).

Table 1. *Estimations of phlorrhizin in dog plasma to which different amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Lambrechts' method
0	0	0·04
0·06	0·04	0·09
0·12	0·13	0·16
0·23	0·23	0·26
0·47	0·48	0·51
0·70	0·68	0·72
0·94	0·95	0·97
1·17	1·19	1·19
1·41	1·41	1·44
1·64	1·66	1·66
1·88	1·84	1·89

Estimation of phlorrhizin in bile. Jenner & Smyth (1957) have shown that when phlorrhizin is administered to animals, large amounts appear in the bile. For this reason, relatively high concentrations of phlorrhizin were made up in dog bile. The bile was obtained by cannulation of the common bile duct after ligation of the cystic duct. Although bile contains substances which have a high extinction value at 285 mμ, it is possible to use u.v. spectroscopy for estimation of phlorrhizin in bile since because of the large amounts of phlorrhizin expected the bile can be greatly diluted. Table 2 gives figures for the amounts of phlorrhizin in bile as estimated by u.v. spectroscopy and by the hydrogen peroxide reaction.

Estimation of phlorrhizin in urine. It is not possible to use u.v. spectroscopy for urine unless this is combined with a chromatographic separation of phlorrhizin from other substances which give high extinction values in the u.v. range. Solutions of phlorrhizin in dog urine were made up and estimated both by the hydrogen peroxide method and the chromatographic separation combined with u.v. spectroscopy. The results are given in Table 3.

Table 2. *Estimations of phlorrhizin in dog bile to which various amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Spectroscopic method
0	0	0·02
2·60	2·52	2·54
3·86	4·10	3·78
5·05	4·87	4·89
6·54	6·54	6·65
7·71	7·92	7·67
8·94	8·88	9·03

Table 3. *Estimations of phlorrhizin in dog urine to which different amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Chromatographic method
0	0	0·02
0·04	0·02	0·07
0·09	0·08	0·09
0·18	0·18	0·20
0·27	0·27	0·29
0·54	0·53	0·57
0·81	0·81	0·82
1·08	1·06	1·08
1·35	1·37	1·32
1·89	1·85	1·84

Specificity of the reaction. The reaction has been tested with solutions in borate buffer of phloretin, phlorin, phloretic acid and phloroglucinol. No colour is obtained with any of these substances. The absence of reaction with phloretin is of particular interest, since considerable doubt exists as to whether phlorrhizin or its aglucone is responsible for the biological activity, and hitherto no simple method has been available for distinguishing between the two substances.

Interfering substances. Any substance which reacts with hydrogen peroxide will interfere with the reaction. Such substances, e.g. glucose and ascorbic acid, in the amounts in which they are likely to be present in biological fluids, are not extracted in sufficient quantities to interfere with the reaction. All the phlorrhizin derivatives are extracted with butanol, but of these phloroglucinol is the only one which interferes with the reaction, possibly because of its strong reducing properties. It can, however, readily be distinguished by u.v. spectroscopy, and if necessary can be separated by the chromatographic procedure described.

SUMMARY

A simple colorimetric method is described for the estimation of phlorrhizin in biological fluids. The method appears to be highly specific for phlorrhizin, and is not given by any phlorrhizin derivative which has been tested.

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Glucuronide Synthesis in Foetal Liver and Other Tissues

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Glucuronides are synthesized in adult liver by enzymic transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPglucuronic acid) to an acceptor (Dutton & Storey, 1954; Storey & Dutton, 1955), which may be from a wide range of substances (Isselbacher & Axelrod, 1955; Dutton, 1956; Axelrod, Inscoe & Tomkins, 1957); other adult tissues, notably kidney and gastrointestinal tract, also form glucuronides and the mechanism there is possibly the same (Dutton, 1958*a*).

In liver slices from young mice and foetal rabbits synthesis of *o*-aminophenyl glucuronide appears to be low or absent (Karunairatnam, Kerr & Levvy, 1949; Hartiala & Pulkkinen, 1955). Investigation of this deficiency seemed important, especially since identification of the 'direct' bilirubin in the van den Bergh reaction as bilirubin diglucuronide (Schmid, 1956; Talafant, 1956; Billing, Cole & Lathe, 1957), and the implication of UDPglucuronic acid and the microsomal enzyme [variously termed glucuronyl or glucuronosyl transferase (Levvy, 1956; Isselbacher, 1956) or uridine diphosphate-transglucuronylase (Dixon & Webb, 1958)] in the formation of this conjugate (Lathe & Walker, 1957; Schmid, Hammaker & Axelrod, 1957; Grodsky & Carbone, 1957).

It was necessary to show whether foetal liver had lower levels than adult liver of enzyme, nucleotide or both, or whether these values were at adult level and apparent deficiency arose from inhibition of the enzyme or hydrolysis of the conjugate. Synthesis in liver slices reflects the formation of both conjugate and UDPglucuronic acid; in liver homogenates fortified with the nucleotide only the final glucuronyl transference occurs. It therefore seemed of value to follow synthesis of *o*-aminophenyl glucuronide in both tissue preparations through foetal into adult life, and to supplement by more direct measurement of levels of liver UDPglucuronic acid. Other foetal tissues would also need to be examined for glucuronide synthesis.

The present work indicates that the relative deficiency in foetal liver is due to low levels of both enzyme and nucleotide, and illustrates their gradual increase up to and after birth. Conjugation below adult level is also shown in foetal kidney, but apparently not in foetal stomach or intestine, under the conditions employed. Evidence was obtained from guinea-pig, mouse and human tissues. Preliminary accounts of this work have already appeared (Dutton & Greig, 1957; Dutton, 1958*a*, *b*).

EXPERIMENTAL

Materials

Acceptor substrates. These were resublimed *o*-aminophenol (OAP), recrystallized (−)-menthol and recrystallized *p*-aminobenzoic acid.

Uridine diphosphate glucuronic acid. Boiled liver extract or barium–ethanol fractionated nucleotide was used with, for confirmation, either chromatographically pure preparations (Storey & Dutton, 1955) or 'uridine diphosphoglucuronic acid' (98–100% quoted purity, ammonium salt) from Sigma Chemical Co., St Louis, Mo., U.S.A. In all cases the amount added was checked to ensure that it was not a limiting factor for conjugation during the incubation period of 30 min.

Other additions. β-Glucuronidase was prepared by the method of Kerr, Graham & Levvy (1948), with incubation for conjugate hydrolysis at pH 5·1 for 3 hr. at 37°. Saccharo-1:4-lactone was prepared in solution according to Levvy (1952). D-Glucurone and potassium glucuronate dihydrate were gifts from the Corn Products Refining Co., New York, U.S.A. Glucose 1-phosphoric acid (dipotassium salt) was from British Drug Houses Ltd.

Animals

Mice. Mice were from a mixed colony and of either sex; litters were segregated. Adults were killed by cervical dislocation, young mice by decapitation. Required organs were rapidly removed to crushed ice, those from very young litter mates being pooled if necessary.

Guinea pigs. These were from a mixed colony. Adults and young were killed by a sharp blow on the head, and organs or foetuses placed in crushed ice, required foetal tissues being removed at once. Foetal ages were estimated from the weight and length of the excised litter, the data of Draper (1920), Ibsen (1928) and Bell (1941) being used. In the group '9 weeks–birth' are included those litters where maternal behaviour and their own physical development indicated imminent parturition.

Human. The human foetuses, each between 3 and 4 months old, were normal and obtained therapeutically; immediately on excision the foetus was placed in crushed ice and the placenta, liver and kidneys were removed and cooled before being rapidly transported to the Laboratory. Allowance for this additional 15–20 min. in ice was made by suitable controls.

Methods

Synthesis with sliced tissue. Tissues were sliced by the method of Cohen (1957), except gastrointestinal tract, which was cut into suitable strips, care being taken to remove loose fat or muscle and to open out the gut. For synthesis of *o*-aminophenyl glucuronide the method of Storey (1950) was used, the slices being shaken for 1 hr. at 37° in sulphate-free Ringer bicarbonate soln. (Krebs & Henseleit, 1932), with CO_2+O_2 (1:19) as gas phase; 0·23 mM-OAP and mM-ascorbic acid were present. Triplicate determinations were made where possible. Tissues were dried at 110° for 2 hr. before weighing. Glucose, glucurone and potassium glucuronate were added in some experiments, to a final concn. of 20 mM. Where menthyl glucuronide was to be measured the procedure, after removal of slices, was as for the corresponding homogenate preparation (Dutton & Storey, 1954).

Synthesis with homogenized tissue. The methods were described previously, for OAP and (−)-menthol (Dutton & Storey, 1954; Storey & Dutton, 1955) and for *p*-aminobenzoic acid (Dutton, 1956). With OAP as substrate, each flask contained 33 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·5, 10 mM-$MgCl_2$, UDPglucuronic acid preparation (see under Materials; when pure, a concn. of 0·1 mM was present), 0·14 mM-OAP, 0·76 mM-ascorbic acid and 0·5 ml. of 10% (w/v) tissue homogenate, in a total volume of 3 ml. Incubation was for 30 min. in air at 37°.

Levels of uridine diphosphate glucuronic acid. The method used for estimation gave a reasonably accurate comparison of the amounts of UDPglucuronic acid present in foetal and adult tissue. Immediately on death the liver was excised and cooled for 1 min. in ice; 1 g. was rapidly weighed, and dropped into 4 ml. of boiling water and boiled for 2 min.; the mixture was cooled, the tissue was disrupted by the homogenizer and the suspension centrifuged at 2000 *g* for 5 min. Flasks had been set up which contained homogenate-reaction medium complete but for UDPglucuronic acid, and 0·5 ml. of the supernatant was then added to them as a source of the nucleotide. Corresponding flasks were run without this addition, and the increase in synthesis of *o*-aminophenyl glucuronide due to the added extract could be determined. With very small livers, less than 1 g. was used and the volume of water was reduced accordingly. A check was always made to ensure that homogenate concentration was not a limiting factor.

Controls. As well as those mentioned above, controls were run in each experiment as a check on the continued suitability of technique and media; for example, foetal preparations were always accompanied by those from an adult. When the choice arose, foetal tissues were exposed least to the chance of accidental loss of activity; and, if necessary, adult tissue was manipulated in amounts small enough to correspond with the foetal samples.

RESULTS

Synthesis of o-aminophenyl glucuronide in young mouse liver

Slices. For sliced tissue, the results of Karunairatnam *et al.* (1949) were confirmed. Formation of *o*-aminophenyl glucuronide by livers of 1- to 5-day-old mice was negligible when compared with that in corresponding adult preparations; about 50% of the adult level was reached in 16–20 days, and adult values were attained by 4 weeks of age (Fig. 1).

Homogenates. Although negligible conjugation occurred also in UDPglucuronic acid-fortified homogenates of liver from day-old mice, the subsequent rise in activity seemed more rapid than with slices, often reaching 50% of the adult value by 7 days. Considerable variation between, but not within, litters was noticed; the degree of glucuronide formation appeared to correspond with body weight rather than the age, and since smaller litters (five to six) were heavier for their age than larger ones (12–14) the typical familial difference shown in Fig. 1 is explicable.

Levels of UDPglucuronic acid. Tentative experiments suggested that liver UDPglucuronic acid was at a lower level in young mice than in adults, but the nucleotide is very labile in tissues and results obtained with such small and easily damaged livers required further confirmation.

Synthesis of o-aminophenyl glucuronide in foetal and young guinea-pig liver

Because of its more convenient foetal size, the guinea pig was used in subsequent studies. Since the young are born in an advanced state, investigation was begun on the early foetus.

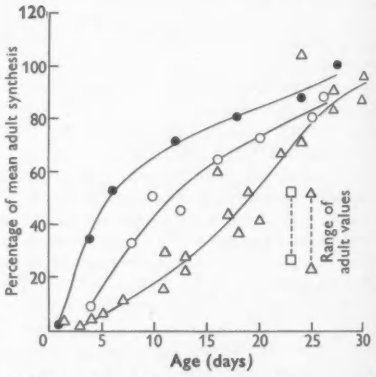

Fig. 1. Glucuronide synthesis by mouse-liver preparations at various ages, expressed as percentage weight of *o*-aminophenyl glucuronide formed, the mean adult value from 25 animals (for slices) or from 30 animals (for homogenates) being taken as 100. Sliced tissue: △, shaken for 1 hr. at 37°, gas phase CO_2+O_2 (1:19), with 0·23 mM-*o*-aminophenol and mM-ascorbic acid. Homogenates: ●, from a small litter; ○, from a large litter; □, from adults. Homogenate (0·5 ml.) was present in a total volume of 3 ml. containing 33 mM-tris buffer, pH 7·5, 10 mM-$MgCl_2$, UDPglucuronic acid (see text), 0·14 mM-*o*-aminophenol and 0·76 mM-ascorbic acid, incubated in air at 37° for 30 min. All values are means of duplicate determinations.

Slices. Formation of *o*-aminophenyl glucuronide could not be shown in liver slices from foetuses estimated at 5–6 weeks' gestation, and the amount of conjugation in later specimens increased slowly. Glucose, glucose 1-phosphate, glucurone or glucuronate (all 20 mM) did not increase the extent of synthesis. Just before birth foetal slices had, on the dry-weight basis employed, about 10% of the adult conjugation. After birth the increase was more rapid, reaching 30–40% at 4 days, as shown in Table 1. It should be noted that in this and subsequent tables the division of foetal ages into groups is one of convenience; since increase in extent of conjugation is progressive with age, the range quoted in each foetal group reflects this and not necessarily experimental error or random individual variation.

Homogenates. In UDPglucuronic acid-fortified homogenates from early foetal guinea-pig liver, formation of *o*-aminophenyl glucuronide was very low, suggesting a deficiency of UDP-transglucuronylase activity, compared with the adult. Just before birth the level had risen to about 40% of the mean adult figure, and after 1–2 days to about 60%, adult values being reached by 5 days of age (Table 2).

A few experiments with microsomal preparations (see Dutton, 1956) confirmed that foetal liver conjugated less *o*-aminophenol than did the adult tissue, but the lengthy procedure was unsuitable for this work. In agreement with the observations made on young mice, fortified homogenates of foetal guinea-pig liver showed a level of glucuronide formation nearer to that of the adult than did

Table 1. *Glucuronide synthesis by guinea-pig tissue slices at various ages*

Incubation was for 1 hr. at 37° in sulphate-free Ringer bicarbonate soln. containing 0·23 mM-*o*-aminophenol (OAP) and mM-ascorbic acid; 20 mM-glucose was present where indicated; gas phase, $CO_2 + O_2$ (1:19). Individual results are the means of duplicate or triplicate determinations. In this and subsequent tables, where results are not individually reported, means are given with ranges in parentheses and the number of animals in superior figures; – means not determined.

		OAP conjugated (μg./50 mg. dry wt. of tissue/hr.)		
Age	Glucose	Liver	Kidney	Stomach
Foetus, 5–7 weeks	–	0, 0, 0·5	0·6, 0·8	2·2, 1·9
	+	0, 0·4, 0·5	0·8, 1·2, 1·3	6·5, 4·7, 3·1
Foetus, 7–8 weeks	–	0·6, 1·2, 1·5	1·0, 1·5	—
	+	0·6, 1·8, 2·0	1·8, 3·2	10·6, 8·5
Foetus, 8–9 weeks	–	1·5, 2·8, 5·0	2·6, 3·1	—
	+	1·8, 2·3	3·2, 3·4, 3·8	6·0, 5·5, 2·3, 1·7
Foetus 9 weeks–birth	–	1·6, 2·8, 3·0	3·3, 4·5	—
	+	1·5, 3·5, 3·8	4·1, 5·6, 6·7	—
Young, 1 day	–	4·0	—	—
	+	5·0	5·5	5·0
Young, 2 days	–	11·0, 14·0	—	—
	+	—	12·8	—
Young, 4 days	–	17·0, 24·0	—	—
	+	—	—	—
Adult	–	35·1 (26·5–42·0)[17]	19·3 (15·1–26·0)[4]	1·2 (0·8–1·5)[8]
	+	26·0 (22·0–30·5)[3]	33·2 (31·5–35·6)[7]	3·5 (1·5–9·2)[7]

Table 2. *Glucuronide synthesis by guinea-pig liver homogenates at various ages*

Each flask contained 3 mM-tris buffer, pH 7·5, 10 mM-$MgCl_2$, UDPglucuronic acid preparation (see text), aglycone and 0·5 ml. of 10% (w/v) liver homogenate in a total of 3 ml. Aglycone for (*a*) was 0·14 mM-*o*-aminophenol (with 0·76 mM-ascorbic acid); for (*b*), 6·4 mM-(–)-menthol; for (*c*) 0·14 mM-*p*-aminobenzoic acid. Incubation was in air for 30 min. at 37°. Individual figures quoted are the means of duplicate determinations.

	Aglycone conjugated (μg./50 mg. wet wt. of liver/30 min.)		
Age	(*a*)	(*b*)	(*c*)
Foetus, 5–7 weeks	0·5, 0·6, 0·8, 1·2, 1·8	1·0	0
Foetus, 7–8 weeks	2·1 (1·6–2·5)[10]	—	—
Foetus, 8–9 weeks	3·1 (2·2–3·9)[6]	6·3	0·8
Foetus, 9 weeks–birth	4·2, 4·8, 5·3, 6·7	10·0	—
Young, 1 day	3·9, 7·2, 8·5	—	—
Young, 2 days	5·6, 9·5	—	—
Young, 5 days	12·6	—	—
Adult	12·2 (7·9–14·5)[30]	18·1, 16·2, 19·1	5·8, 8·1

slices of a corresponding age or, more strikingly, than did slices from the same foetal animal.

Levels of uridine diphosphate glucuronic acid. The above phenomenon suggested a low level, or poor utilization, of UDPglucuronic acid in intact foetal liver. The amount of this nucleotide in foetal, as compared with that in identically prepared adult, boiled liver extracts was measured by the increase in conjugation each afforded when added to an unfortified homogenate of adult guinea-pig liver (Table 3). In spite of the inexactitude of this method the differences were outstanding. It was obvious that guinea-pig liver did not gain its high content of UDPglucuronic acid until birth, and it was thus reasonable to suppose that this lack superimposed on low activity of UDP-transglucuronylase accounted for the poorer synthesis in slices.

Weight of foetal guinea-pig liver

It was noticed that livers of guinea-pig foetuses of some 8 weeks onwards became enlarged, pale and fatty. According to Imrie & Graham (1920), such livers contain some 15% by weight of fatty acids, these disappearing a few days after birth. The fat would be included in both the wet- and dry-weight methods used in this work, and would interfere significantly during the last stages of gestation.

Total body and total liver wet weights were determined, together with the final weights of 1 g. of wet liver dried at 110° for 5 hr. Total wet and total dry liver weights were then separately expressed as a percentage of the body weight; for six adults of both sexes these values were in the range 3·3–5·3% and 0·9–1·2% respectively. From the data of Bogdanovitch & Man (1938) the mean figure for the former value was 3·1%, for thirty animals of both sexes, 1–35 months old. Six foetuses from separate litters at 9 weeks–full term, and one animal at a day old, were examined similarly. The wet-liver weight ranged from 5·0 to 7·4 (mean 5·7)% of body weight and the dry-liver weight from 1·9 to 2·5% (mean 2·1, one not determined). Two earlier foetuses (6–8 weeks), as expected, yielded results about the adult level, as did an animal 5 days old.

In the late foetus therefore both the ratios (wet-liver weight:body weight) and (dry-liver weight:body weight) are higher than in the adult, up to roughly two and three times respectively, this estimate being based on extreme examples of results so far obtained. This would have the effect of diminishing that discrepancy noted above between synthesis in slices and in homogenates of the same foetal liver. Five days after birth the livers were visibly similar to those of the adult; the extra fat, according to Imrie & Graham (1920), has disappeared and this is noticeable in the fallen liver (dry weight:body weight) ratios.

Further evidence for low levels of uridine diphosphate-transglucuronylase

Although low activity of UDP-transglucuronylase was noted in homogenates of foetal liver, this might have been due, not to a smaller amount of the enzyme itself, but to its inhibition or to destruction of the conjugate by the known high level of β-glucuronidase in young tissues (Karunairatnam *et al.* 1949). To test the first supposition, various amounts of homogenate of foetal liver were added to a similar preparation of adult liver (Table 4). The conjugation observed in such a mixture, where not increased additively, shows an insignificant fall.

Table 3. *Levels of uridine diphosphate glucuronic acid in guinea-pig liver at various ages*

Conditions were as described in Table 2 (*a*), except that UDPglucuronic acid preparation was replaced by 0·5 ml. of extract obtained by boiling 1 g. (wet wt.) of liver sample in 4 ml. of water (see text); adult guinea-pig liver was used as enzyme source. The resulting weight of conjugated OAP was subtracted from that measured in control flasks without any added source of UDPglucuronic acid. Individual results are the means of duplicate determinations.

Age	Increased OAP conjugation due to added extract (μg./50 mg. wet wt. of liver/30 min.)
Foetus, 5–7 weeks	0·1, 0·2
Foetus, 7–8 weeks	0·4, 0·5, 0·8
Foetus, 8–9 weeks	0·5, 0·7, 0·8, 0·8
Foetus, 9 weeks–birth	0·4, 0·9, 0·9, 1·2, 1·3
Young, 1 day	1·7, 1·8
Young, 2 days	2·4
Young, 5 days	5·9
Adult	5·5 $(4·2–9·1)^{20}$

Table 4. *Effect of various additions on glucuronide synthesis by homogenates of foetal or adult guinea-pig liver*

Media and incubation were as described in Table 2, except where indicated. Boiled saccharate soln. was used as source of saccharo-1:4-lactone (see text). Figures are means of duplicate determinations. *A* and *C* were homogenates of livers of foetuses of 5–7 weeks and of 7–8 weeks respectively. *B* and *D* were homogenates of adult livers.

Liver homogenate present	OAP conjugated (μg./30 min.)
A, 0·5 ml.	0·8
B, 0·5 ml.	11·1
A, 1·0 ml.	1·3
B, 1·0 ml.	18·7
A, 0·5 ml. + *B*, 0·5 ml.	10·8
A, 1·0 ml. + *B*, 0·5 ml.	9·0
C, 0·5 ml.	2·5
D, 0·5 ml.	10·5
C, 0·5 ml. + *D*, 0·5 ml.	12·0
C, 0·5 ml. + boiled 0·15 mM-saccharate	2·2
C, 0·5 ml. + boiled 1·5 mM-saccharate	1·8
D, 0·5 ml. + boiled 0·15 mM-saccharate	10·0

Table 5. *Glucuronide synthesis in human foetal tissues*

Conditions were as described in Table 1 for sliced tissue, as in Table 2 (*a*) for homogenates; UDPglucuronic acid levels were estimated by a method similar to that described in Table 3. *A* is a human foetus of 3·5–4 months; *B*, of 3–3·5 months. Tissues from mouse *X* were treated exactly as those from *A* and *B* (see text); those from mouse *Y* were used immediately after death. Figures quoted for adult-human liver were communicated by Dr I. D. E. Storey. Values are means of duplicate determinations.

Tissue	OAP conjugated (μg./50 mg. dry wt. of slices/hr.)	OAP conjugated (μg./50 mg. wet wt. of homogenate/hr.)	UDPglucuronic acid (% of that in equal wt. of fresh adult-mouse liver)
Placenta from *A*	0	0	10
Kidney from *A*	0·6	—	—
Kidney from *B*	0·5*	—	—
Liver from *A*	0·3	0·1	12
Liver from *B*	0·2	0·2	10
Adult human liver	25·0, 29·5, 27·0	—	—
Liver from mouse *X*	24·1	5·0	62
Kidney from mouse *Y*	5·0, 10·2*	—	—
Liver from mouse *Y*	28·2	5·8	100

* Glucose present (20 mM).

and indicates that an inhibitor of UDP-transglucuronylase activity is unlikely in foetal liver. It also suggests that low synthesis is not due to rapid destruction of UDPglucuronic acid, as may happen in kidney and gut homogenates (Dutton & Greig, 1957). Saccharo-1:4-lactone, the specific inhibitor for β-glucuronidases (Marsh & Levvy, 1958), when added to homogenates of foetal liver did not increase their conjugating power. Thus hydrolysis of formed glucuronides is not responsible for their low yield in preparations of foetal liver. UDP-transglucuronylase itself would therefore seem to be at a low level in foetal liver.

Synthesis of other glucuronides in guinea-pig liver

Because of the rapid, sensitive and specific method available for this substrate, the conjugation of *o*-aminophenol only was followed in detail through foetal development. Evidence was obtained that the glucuronides of (−)-menthol and of *p*-aminobenzoic acid were formed to very small extent, if at all, in the early foetal guinea-pig liver, increasing later as with the previous substrate (Table 2). *p*-Aminobenzoyl glucuronide is an ester-type glucuronide, synthesized in liver in the same way as the ether-type glucuronide of *o*-aminophenol (Dutton, 1956).

Synthesis of o-aminophenyl glucuronide in other guinea-pig tissues

Foetal kidney. Since slices of guinea-pig kidney formed *o*-aminophenyl glucuronide at a rate, when glucose was present, comparable with that of liver, this organ was studied in the foetus (Table 1). Conjugation was low, and though increased with glucose to levels above those in corresponding foetal-liver slices, it remained below adult value until after birth. Glucuronide formation in crude homogenate of adult kidney is small (Dutton & Greig, 1957), so that its low level in foetal homogenates was not profitable to investigate under these conditions.

Placenta and uterus. The early foetal guinea-pig being unable to form glucuronides to any extent through its own liver and kidney, the maternal uterus and placenta were examined; neither gave evidence of significant formation of *o*-aminophenyl glucuronide at different foetal ages.

Other foetal tissues. Considerable synthesis of glucuronide occurs in slices of stomach and intestine of various adult animals, provided that glucose is present (Zini, 1952; Shirai & Ohkubo, 1954; Hartiala, 1955; Schachter, Kass & Lannon, 1958; Dutton, 1958*a*), though much variation in activity is encountered. By several methods it has been proved that this tissue forms the glucuronide of *o*-aminophenol in the guinea-pig foetus. On a dry-weight basis, the activity seems to be at or above adult level in foetal stomach from at least the sixth week, and to fall gradually (Table 1). Because of the use of entire stomach-wall strips instead of the active but not easily separable mucosa, this fall may merely reflect the increasing inclusion with growth of non-conjugating tissue. Evidence for the mechanism of glucuronide synthesis at extra-hepatic sites such as stomach and kidney will be presented more fully elsewhere. Stomach is the most active early foetal tissue yet examined, slices of lung, spleen or brain having negligible conjugating power under the conditions employed.

Synthesis of o-aminophenyl glucuronide in human placenta, foetal liver and foetal kidney

Results obtained with two normal human foetuses of 3–4 months and with human placenta of the same period are shown in Table 5, together with results observed in control preparations from

adult-mouse tissues, both fresh and subjected to the same delay as the human specimens. Adult human-liver slices form the glucuronide at roughly the level of those from adult-mouse liver (Dr I. D. E. Storey, personal communication). The loss of activity at 0° consequent on delay did not affect the mouse preparations to a great extent, and the low values recorded for the human examples must therefore have been due to their foetal state. This bears out the results obtained with mouse and guinea pig, by showing negligible formation of *o*-aminophenyl glucuronide in early foetal liver and kidney, or in placenta, traceable in the liver to low levels of UDP-transglucuronylase activity and of UDPglucuronic acid.

DISCUSSION

It follows from previous work (Dutton & Storey, 1954; Storey & Dutton, 1955) that in liver homogenates the step

Acceptor + UDPglucuronic acid → acceptor glucuronide + UDP

only is being studied, whereas in slices the synthesis (see Strominger, Maxwell, Axelrod & Kalckar, 1957) of the nucleotide from glycogen or α-glucose 1-phosphate also takes place. Since excess of UDPglucuronic acid is supplied to homogenate preparations, their synthetic power may be largely a measure of UDP-transglucuronylase activity, whereas that of slices, being dependent on endogenously formed nucleotide, may reflect more accurately the position in the intact animal. Any striking difference between these synthetic performances is due possibly to lack of availability of UDPglucuronic acid for the transference reaction; actual levels of the nucleotide in liver would be deduced from the behaviour of the boiled extract.

In the early foetal guinea-pig liver it is clear that formation of the glucuronides studied is negligible and this arises from very low levels of both enzyme and nucleotide. This condition is seen also in the human foetus of 3–4 months, and it is probable that the lack of synthesis of *o*-aminophenyl glucuronide observed in early foetal rabbit-liver slices (Hartiala & Pulkkinen, 1955) is from the same cause. Though foetal-mouse liver has not been studied a low level of enzyme may be presumed present, for this is certainly the case with the organ from new-born animals. Glucuronide synthesis below adult level has been shown in guinea-pig and human foetal-kidney slices, and has been reported from similar preparations in the young mouse (Karunairatnam *et al.* 1949).

It would thus seem that negligible amounts of UDP-transglucuronylase and UDPglucuronic acid are typical of the first two-thirds of foetal life in the species studied, and this is associated with low formation of *o*-aminophenyl glucuronide in the kidney. There is considerable glucuronide synthesis in the stomach, and some in the gut, of early foetal guinea pig, but because of the shortcomings in its liver and kidney conjugatory function the foetus of this age is relatively poorly equipped, compared with the adult, for 'detoxication' through glucuronide formation. Since the placenta and uterus seem ineffective in this role, the early foetus must largely rely on 'detoxication' by the maternal liver, kidney and gastrointestinal tract.

This dependence may persist until birth in the mouse, but is to some extent ameliorated by full term in the guinea pig, whose greater development at birth possibly accounts for the species difference. Apparently UDP-transglucuronylase activity increases more rapidly in foetal guinea-pig liver than does UDPglucuronic acid, and this is reflected in the slow development of the glucuronide-synthesizing system in slices. In the full-term guinea pig the enlarged fatty foetal liver must be taken into account. From the maximum increases in liver wet and dry weight: body weight ratios, UDP-transglucuronylase has increased at full term to

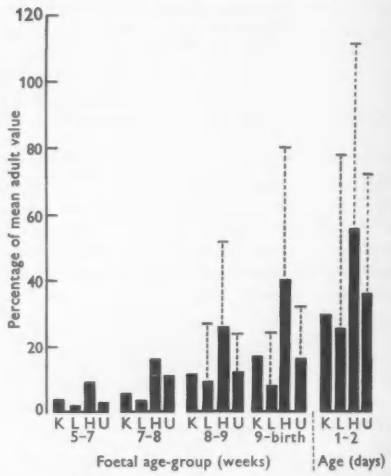

Fig. 2. Synthesis of *o*-aminophenyl glucuronide and levels of UDPglucuronic acid in preparations of guinea-pig tissue at various ages, expressed as percentage of the mean value found in adult tissue. K, Kidney slices; L, liver slices; H, liver homogenate; U, UDPglucuronic acid estimated as in text. Filled-in columns show mean percentages as determined for 50 mg. dry wt. (K, L) or wet wt. (H, U) of tissue; broken-line extensions illustrate the mean percentage attainable in total liver if maximum allowance for its fatty enlargement in the late foetus is assumed (see text).

about 80–100 % of the mean adult value, UDPglucuronic acid to about 60 %, and conjugation in slices to about 30–40 %. In spite of the complexity introduced by fatty infiltration, the pattern in the total liver is still of a rather higher enzyme than nucleotide level, with a poorer synthesis in slices than in fortified homogenates (Fig. 2); however, whereas low formation of the nucleotide probably accounts for the latter discrepancy in foetuses up to some 8 weeks, after this period the greater interference by fat in the dry weights recorded for slices would diminish the observed effect of any increased endogenous supply of UDPglucuronic acid.

Four to five days after birth these late-foetal characteristics have almost disappeared in the guinea pig, and both liver UDPglucuronic acid and glucuronide synthesis in slices are at adult level. Recent work by Schachter *et al.* (1958) would confirm this, inferring that in slices of young guinea-pig liver synthesis of both ether- and ester-type glucuronides of salicylic acid increased from 'minimal' values at birth to 50–80 % of adult figures after 3–4 days.

It is reasonable to suppose that since the mechanism studied above is a general one for glucuronide formation in liver, then its deficiency would affect the synthesis of other examples than those reported here. Particular interest would be attached to bilirubin diglucuronide. It is known that UDPglucuronic acid transfers its glucuronic acid to bilirubin in the livers of the human infant and adult, and of adult rats (Lathe & Walker, 1957; Grodsky & Carbone, 1957; Schmid *et al.* 1957). That the same microsomal enzyme is involved as the one studied here is probable from the reduced synthesis of both phenyl and bilirubin glucuronides in congenitally jaundiced adult humans and rats (Axelrod, Schmid & Hammaker, 1957; Carbone & Grodsky, 1957). The rats had normal UDPglucuronic acid levels and so in their case probably, and in the other possibly, the enzyme was deficient. Lathe & Walker (1957) report a deficiency in enzymic transfer of glucuronic acid to bilirubin in the jaundiced new-born human, and the recent work of Brown, Zuelzer & Burnett (1958), and of Lacson & Waters (1957), cited by Faber *et al.* (1958), indicates that in the guinea pig, and possibly in man, the system synthesizing bilirubin glucuronide develops in foetal and neonatal life similarly to the one forming *o*-aminophenyl glucuronide studied above.

From these results it seems that an enzymic deficiency in glucuronyl transfer, accompanied by low UDPglucuronic acid levels, is a foetal characteristic in the species studied, and hence it may be that certain types of neonatal jaundice are due to persistence of this foetal metabolism, particularly in premature infants. Where lack of UDPglucuronic acid is a limiting factor, it is possible, though unlikely, that its administration, or that of glucose, might prove of use; attempts to induce formation of glucuronide by treatment with glucuronidogenic drugs could lead, in deficiency of UDP-transglucuronylase, to competition with bilirubin for reaction at the few enzyme molecules available.

SUMMARY

1. Low *o*-aminophenyl glucuronide synthesis in foetal liver of guinea pig and man and in neonatal mouse liver has been shown to be due to low levels of uridine diphosphate glucuronic acid and of uridine diphosphate-transglucuronylase (glucuronyl transferase) activity, the enzyme itself probably being present only in small amount.

2. In early foetal guinea-pig liver both these levels are negligible, but increase towards full term. Relatively greater conjugation was observed in homogenates fortified with uridine diphosphate glucuronic acid than in corresponding slices; the bearing of this phenomenon on formation of the nucleotide and on fatty infiltration of foetal liver is discussed. After birth, guinea-pig liver rapidly assumes the glucuronide-synthesizing capacity of the adult. A somewhat similar pattern is described for the infant mouse.

3. Synthesis below adult levels also of (−)-menthyl glucuronide and of the ester-linked *p*-aminobenzoyl glucuronide occurs in homogenates of foetal guinea-pig liver.

4. *o*-Aminophenyl glucuronide synthesis in early foetal kidney is negligible but increases towards full term, being at birth, when fortified with glucose, higher than in corresponding liver slices, though still below adult level.

5. The most active site of glucuronide synthesis so far examined in the early foetus of the guinea pig is the stomach.

6. 'Defective' glucuronide formation in liver and kidney appears to be a foetal characteristic of the species studied, and its relation to bilirubin conjugation in neonatal jaundice is discussed.

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Incorporation of Amino Acids into some Subcellular Fractions of Hepatic Tissues of the Rat

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After the injection of an isotopically labelled amino acid there is a marked difference in the rate at which the protein of the various subcellular constituents of the liver cells becomes labelled. This was first shown by Hultin (1950) with chicks, then by Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1950) for guinea pigs, and later by Keller, Zamecnik & Loftfield (1954) for rats. When slices of rat liver are incubated with a labelled amino acid and subsequently homogenized, the distribution of radioactivity in the different subcellular fractions is very similar to that in the experiment *in vivo* (Campbell, Greengard & Jones, 1957). If homogenates of liver are incubated with a radioactive amino acid and a suitable energy source, the distribution of radioactivity is still similar to that in the intact cell (Siekevitz, 1952). Thus a certain characteristic of the amino acid-incorporating system is maintained in the disrupted cells, suggesting that it might be possible to study the regulation of protein synthesis in subcellular preparations.

In view of the above considerations it was naturally of interest to continue our experiments, designed to elucidate differences between protein synthesis in the normal and malignant liver cell, on cell-free systems. As in our previous studies, liver tumours have been induced by feeding 4-dimethylaminoazobenzene to rats. In order to make comparisons with a rapidly growing normal tissue, preparations from regenerating liver have also been used.

Since the microsomal fraction of the liver cell incorporates amino acids more rapidly than any of the other subcellular fractions so far studied, emphasis, in the present experiments, has been placed on this fraction. Zamecnik & Keller (1954) were the first to demonstrate the incorporation of

amino acids into the protein of isolated microsomes. Their system, in which the level of adenosine triphosphate is maintained by an added source, has the advantage that differences due to the ability of the different tissues to generate energy-rich phosphate are eliminated. Such microsomal preparations have been used therefore in the present experiments. The effect of the various subcellular fractions on the ability of each to incorporate amino acids has also been studied.

A preliminary report of some aspects of this work has already appeared (Campbell & Greengard, 1957).

MATERIALS AND METHODS

Chemicals. The dipotassium salt of adenosine triphosphoric acid (ATP) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. and neutralized with KOH. The barium silver salt of phosphoenolpyruvic acid (PEP) was prepared by the method of Ohlmeyer (1951). A solution of the free acid was prepared shortly before use and was adjusted to pH 7. Glutathione was obtained from The Distillers Co. (Biochemicals) Ltd., Liverpool, and used in a freshly prepared solution (adjusted to pH 7 with KOH).

Radioactive amino acids. Uniformly labelled [^{14}C]-glycine (100 μc $\equiv$ 1·16 mg.), L-[^{14}C]leucine (100 μc $\equiv$ 1·49 mg.) and L-[^{14}C]lysine (100 μc $\equiv$ 1·13 mg.) were supplied by The Radiochemical Centre, Amersham, Bucks.

Tissues. (1) Liver tumours. Tumours were induced in rats by feeding them on a diet containing 0·06% of 4-dimethylaminoazobenzene as described by Campbell (1955). All rats bearing tumours were kept on a normal diet free of the carcinogen for at least 4 weeks before use. (2) Regenerating liver. Rats (150 g. body wt.) were partially hepatectomized by the method of Higgins & Anderson (1931). The regenerating livers were used 44–48 hr. after the operation. (3) Normal liver. This was obtained from rats (150 g. body wt.) fed on the stock diet.

Tissue preparations. In all cases centrifuging took place at approx. 1°. (1) Microsomes. The method used followed closely that described by Zamecnik & Keller (1954). The tissue was homogenized in 2·5 times its weight of a medium containing phosphate buffer (0·01 M-$MgCl_2$, 0·02 M-potassium phosphate (pH 7·8), 0·03 M-$KHCO_3$, 0·025 M-KCl and 0·35 M-sucrose). The tissue suspension was centrifuged at 12 000 *g* for 5 min. The supernatant from this centrifuging was then centrifuged in a Model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 *g*. The surface of the microsomal pellet was washed with 1 ml. of medium and suspended, in 0·4 ml. of medium/g. of original liver, by gentle homogenization in a small all-glass Potter homogenizer. (2) Mitochondria. The method used was based on that of Schneider & Hogeboom (1950). The tissue was homogenized in a Potter–Elvehjem type glass homogenizer in nine times its weight of 0·25 M-sucrose. The debris and nuclei were removed by centrifuging at 500 *g* for 30 min. The supernatant was then centrifuged at 12 000 *g* for 10 min. The supernatant, together with the fluffy layer, was decanted, and the sediment homogenized in a small volume of 0·25 M-sucrose and spun down at 12 000 *g* for 10 min. Before incubation the mitochondria were suspended in medium as described for the microsomes. (3) Nuclei. The tissues were homogenized in an equal weight of 1·45 M-sucrose, followed by a further homogenization in ten times the tissue weight of 0·88 M-sucrose (Palade, 1951). The sediment after centrifuging at 600 *g* for 10 min. was discarded and the supernatant was spun again at this force for 20 min. The sedimented nuclei were stirred with a rod in 0·88 M-sucrose and respun. Before incubation the pellet was suspended in medium as described for the microsomes.

Method of incubation. The above cell fractions, individually or in combination, were incubated under a continuous stream of $N_2 + CO_2$ (95:5) at 37° in a total volume of 1 ml. The incubation mixture contained 2 μmoles of ATP, labelled amino acid, 10 μmoles of PEP, 0·3 ml. of cell sap (i.e. supernatant after centrifuging at 105 000 *g*) and 0·4 ml. of the suspension of the particles in the medium containing phosphate buffer.

The amount of microsomal proteins in each tube was 6–7 mg. The wet weight of tissues yielding this amount of microsomal protein was 1 g. of liver and regenerating liver and 1·5 g. of tumour. The amount of mitochondrial protein per tube was 8–9 mg., obtained from 1·2 g. wet wt. of either normal or regenerating liver or 2·5 g. of tumour. The amount of nuclear protein per tube was 5–6 mg., obtained from 2 g. of either normal or regenerating liver or tumour.

Isolation of protein. If, after incubation, it was necessary to separate the different particulate fractions, this was effected by the fractionation procedure previously described. The proteins were precipitated in aqueous 5% (approx.) trichloroacetic acid. They were then treated with hot trichloroacetic acid and organic solvents according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951).

In order to determine whether the radioactivity was due to radioactive peptides attached to the protein by disulphide bonds, the normal washing procedure was in some cases followed by treatment of the protein with thioglycollic acid, as suggested by Siekevitz (1952). The protein (10 mg.) was suspended by homogenization in 1·5 ml. of an aqueous solution of 6% (w/v) potassium thioglycollate at pH 8·0. After 2 hr. at room temperature 1·5 ml. of 10% (approx.) trichloroacetic acid was added and the sediment was washed with hot (90°) 5% trichloroacetic acid, cold 5% trichloroacetic acid, ethanol and acetone.

Measurement of radioactivity. The proteins were counted at infinite thickness in one of two ways. In the first, the radioactivity was determined on 0·28 cm.^{2} Perspex disks in a Geiger–Müller counter with a mica end-window (2·3 mg./cm.^{2}). In the second, stainless-steel disks of similar dimensions were used in a Tracerlab SC-16 windowless flow counter. In this case the protein was compressed on the disk with a stainless-steel pestle, as described by Busch Simbonis, Anderson & Greene (1956). In a given time a radioactive sample gave four times as many counts in the flow counter as in the mica end-window counter. The background count in the flow counter was also four times that in the window counter. Provided that the counting rate exceeded 50 counts/min. above background a total of at least 1000 counts was collected, in which case the coefficient of variation of the count was less than 10%. If the radioactivity of the sample fell below 50 counts/min. above background the samples were counted for four periods of 5 min. interspersed with background counts. Under these circumstances, samples having a count exceeding 20/min. had a coefficient of variation of the

Table 1. *Comparison of the incorporation of* [^{14}C]*glycine into microsome preparations of normal and regenerating liver*

Microsomes and cell sap (in amounts described under Methods) were incubated anaerobically for 50 min. with 2 μmoles of ATP and 2 μc of [^{14}C]glycine. An equal volume of 10% trichloroacetic acid was then added. The radioactivity of the protein (microsomal + cell sap) is expressed as counts/min. at infinite thickness/0·28 cm.^{2} with the mica end-window counter.

Phosphoenolpyruvate (10 μmoles)	−	+	−	+	+	+
Normal-liver microsomes	+	+	−	−	+	−
Normal-liver cell sap	+	+	−	−	−	+
Regenerating-liver microsomes	−	−	+	+	−	+
Regenerating-liver cell sap	−	−	+	+	+	−
Radioactivity of protein	5	65	34	236	110	135

Table 2. *Comparison of the incorporation of* [^{14}C]*glycine into microsome preparations of normal liver and liver tumour*

For experimental conditions and expression of results see Table 1.

Phosphoenolpyruvate (10 μmoles)	−	+	−	+	+	+
Normal-liver microsomes	+	+	−	−	−	+
Normal-liver cell sap	+	+	−	−	+	−
Liver-tumour microsomes	−	−	+	+	+	−
Liver-tumour cell sap	−	−	+	+	−	+
Radioactivity of protein	8	77	12	18	18	42

count of less than 15% and samples with a count below 20/min. had a coefficient of variation of the count of less than 20%.

RESULTS

Incorporation of amino acid into microsomal protein

When microsome preparations of normal and regenerating liver are compared under the same conditions, those from the regenerating tissue are found to have an enhanced ability to incorporate amino acid. These results are shown in Table 1.

The control value, i.e. the radioactivity of proteins incubated in the absence of PEP, was established in each case and was considered as being due to a non-specific adsorption, although it may partly represent a real incorporation process, utilizing the residual endogenous energy source. It can be seen that this control value is higher for regenerating liver and, since the same amount of protein was incubated with the same quantity of radioactive amino acid, this consistent result cannot have been due to a difference in non-specific adsorption of radioactive amino acid. The significance of this finding, however, is diminished by the fact that when amino acids other than glycine were used and the microsomal protein was separated from the cell sap after incubation, the values for the control, without PEP, were as low as those of normal liver. The table also shows that when microsomes of regenerating liver were incubated with normal cell sap, or normal microsomes incubated in regenerating cell sap, the incorporation of radioactivity, in both cases, was higher than with normal constituents alone. Thus both fractions of the regenerating liver are superior, in this respect, to that of normal liver. Similar results have been obtained independently by Hultin & von der Decken (1957).

Table 3. *Incorporation of* [^{14}C]*lysine and* [^{14}C]*leucine into microsome preparations of liver and liver tumour*

Microsomes and cell sap (in quantities described under Methods) were incubated anaerobically for 50 min. with 2 μmoles of ATP and 1 μc of [^{14}C]amino acid. The microsomes were separated from the cell sap immediately after incubation. The specific activity of the microsomal protein is expressed as counts/min. at infinite thickness/0·28 cm.^{2}, in the windowless flow counter.

	Liver		Liver tumour	
	Control	With PEP (10 μmoles)	Control	With PEP (10 μmoles)
[^{14}C]Leucine	15	204	20	83
[^{14}C]Lysine	33	256	44	109

The microsomes isolated from liver-tumour slices after incubation with [^{14}C]glycine are as active as those isolated from normal liver incubated under similar conditions (Campbell *et al.* 1957). Microsome preparations of liver tumour, however, were only slightly active (Table 2). The combination of tumour microsomes with normal cell fluid did not lead to a higher incorporation. The tumour-cell sap does not lack the ability to stimulate incorporation since, when used with normal microsomes, a significant incorporation can be obtained.

In all experiments of the type reported in Table 2, the energy-dependent uptake of glycine by tumour preparations was consistent, but so small that it could be doubted whether it represented a real

Table 4. *Effect of glutathione on the incorporation of [^{14}C]lysine by normal microsome preparations of liver and liver tumour*

For details of incubation and measurement of radioactivity see Table 3. Glutathione, when used, was added at the beginning of the incubation.

	Normal liver			Liver tumour			
Phosphoenolpyruvate (10 μmoles)	−	+	+	−	+	+	+
Glutathione (μM)	−	−	2·5	−	−	2·5	1·0
Radioactivity of microsomal protein	20	357	250	41	63	97	93

Table 5. *Effect of the nuclear fraction on the incorporation of [^{14}C]amino acids by liver-microsome preparations*

Microsomal preparations were incubated anaerobically for 50 min. with 2 μmoles of ATP, 10 μmoles of PEP and 2 μc of [^{14}C]glycine or 1 μc of [^{14}C]leucine, in the presence and absence of the nuclear fraction in quantities described under Methods. The nuclear fractions were isolated by centrifuging. When glycine was used the microsomal and cell-sap proteins were precipitated together and their radioactivity is expressed as counts/min./0·28 cm.^{2}, at infinite thickness with the mica end-window counter. When leucine was used, the microsomes were separated from the cell sap and the radioactivity of the microsomal protein is expressed as counts/min./0·28 cm.^{2}, at infinite thickness in the windowless flow counter.

Amino acid	Microsomal fraction of	Nuclear fraction present	Radioactivity of protein
[^{14}C]Glycine	Normal liver	None	30
	Normal liver	Liver	11
	Normal liver	Tumour	13
	Regenerating liver	None	136
	Regenerating liver	Regenerating liver	21
	Regenerating liver	Tumour	8
[^{14}C]Leucine	Normal liver	None	294
	Normal liver	Liver	78

incorporation process. It can be seen, however, from Table 3 that by using amino acids which are taken up to a greater extent, by improving the method of counting and by separating the microsomes from the less radioactive cell-sap proteins after incubation, a considerable incorporation into tumour microsomal proteins may be demonstrated.

It can be seen from Table 4 that a 30% increase in lysine incorporation could be obtained by adding glutathione to the incubation medium, whereas the incorporation by normal liver microsomes is inhibited by it. When [^{14}C]leucine was used, instead of lysine, the stimulation by glutathione was smaller and more variable.

Effect of the nuclear fraction on incorporation of amino acid into microsomes

In preliminary experiments it was observed that, when whole homogenates of liver were incubated with a labelled amino acid, the incorporation into the microsomes was much lower than in the above type of microsomal preparation. The possibility that one of the fractions is inhibitory was tested. The nuclear fractions of all the three hepatic tissues under study were not able to incorporate amino acids to any significant extent into their proteins. When the nuclear fractions were mixed with microsomes, in a ratio similar to that in the cell, a nearly complete inhibition of microsomal incorporation resulted (Table 5). When smaller amounts of the nuclear fraction were used, the inhibition was less marked. Since an inhibition of the already very low tumour microsomal incorporation could not be easily detected, the inhibitory action of the tumour nuclear fraction was tested on normal and regenerating liver microsomes and was found to be as marked as that of the other nuclear fractions (Table 5).

Incorporation of amino acid into mitochondria and their effect on microsomal incorporation

The purity of the mitochondrial fraction used in these studies has not been tested; it is probable that it contains small quantities of microsomes. Simpson, McLean, Cohn & Brandt (1957) found that, after repeated washing, the ribonucleic acid content of liver mitochondria was 1 mg./100 mg. of protein and it is not known to what extent this represents contamination by microsomes.

The results given in Table 6 show that when mitochondria, prepared from normal liver, are incubated with [^{14}C]leucine or [^{14}C]glycine together with some cell sap, there is a considerable incorporation of radioactivity. For leucine the activity of the mitochondrial preparations is less than that of the microsomes under similar conditions, whereas for glycine the reverse tends to be true. Moreover, whereas the incorporation of glycine into the

microsomes requires PEP, that into the mitochondria is not significantly effected by it. If the radioactivities of the thioglycollate-treated proteins are compared it will be seen that the results are qualitatively similar for [^{14}C]glycine and [^{14}C]leucine. The reason for this is probably that, in the presence of mitochondria, glutathione containing [^{14}C]glycine is formed which, through an –S–S– linkage, becomes attached to the protein but can be removed by the reducing agent (Peterson & Greenberg, 1952). The radioactivity of the [^{14}C]leucine-labelled proteins and that of the [^{14}C]glycine-labelled microsomal proteins is not much changed after thioglycollate treatment. This suggests that the synthesis of glutathione is largely confined to the mitochondrial fractions.

In order to investigate the effect of mitochondria and microsomes on each other, they have been incubated together in the presence of [^{14}C]leucine. This presents the problem of separating the two fractions after incubation. The mitochondrial fraction isolated by the usual differential centrifuging after incubation contains microsomes which have aggregated during incubation and thus sediment at a low centrifugal force. To test the extent of this contamination, microsomes were incubated with [^{14}C]leucine under the usual conditions and then mixed with mitochondria which had been incubated in the absence of radioactive amino acid. The two fractions were then separated. From the amount of protein in the two fractions and their respective specific activities, it was calculated that 15% of the microsomes sedimented together with the mitochondria. The values given in Table 7 for mitochondrial incorporation in the presence of microsomes were corrected on this basis, i.e. the radioactivity due to the co-sedimented microsomes was subtracted from the measured activity of the mitochondrial protein. The contamination of microsomes by mitochondria is unlikely because of the tendency for particulate fractions, after incubation, to sediment at lower centrifugal forces. In any case this factor has been eliminated in the experiment, the results of which are given in Table 7.

Table 7 thus shows that the mitochondrial incorporation is greater in the presence of microsomes, whereas that of the microsomes is considerably diminished in the presence of mitochondria. It can also be seen from Table 7 that the PEP-free control value for microsomal protein radioactivity is higher in the presence of mitochondria, the

Table 6. *Comparison of the incorporation of amino acids by mitochondria and microsome preparations from normal liver and the effect of treatment with thioglycollic acid on the radioactivity of the proteins*

For details of incubation and isolation of proteins see Table 1. The treatment with thioglycollic acid is explained under methods. The results are given as counts/min. at infinite thickness/0·28 cm.^{2} with the mica end-window counter.

		Radioactivity of proteins			
		Control		With PEP (10 μmoles)	
Amino acid	Fraction	Untreated	Thioglycollate treated	Untreated	Thioglycollate treated
[^{14}C]Leucine	Microsomal	15	14	207	177
	Mitochondrial	15	15	74	64
[^{14}C]Glycine	Microsomal	13	6	50	35
	Mitochondrial	73	15	69	20

Table 7. *Incorporation of [^{14}C]leucine by liver microsome and mitochondria preparations*

Liver microsomes and mitochondria were incubated anaerobically for 50 min. with cell sap (in quantities given under Methods), 2 μmoles of ATP and 1 μc of [^{14}C]leucine. When microsomes were incubated alone, mitochondria which had been incubated in a non-radioactive medium were added to the microsomes immediately after incubation. In order to determine the radioactivity of the different cell fractions, the mitochondria and the microsomes were isolated from the cell sap by differential centrifuging. Results are expressed as counts/min. at infinite thickness/0·28 cm.^{2} in the windowless flow counter.

	Radioactivity of the protein fractions isolated after incubation			
	Microsomal		Mitochondrial	
Fractions incubated	Control	With PEP (10 μmoles)	Control	With PEP (10 μmoles)
Liver microsomal	12	594	—	—
Liver microsomal + mitochondrial	37	285	4	69
Liver mitochondrial	—	—	11	34
Tumour mitochondrial	—	—	17	44

reason probably being that the mitochondrial metabolism, with endogenous substrates, is able to maintain the supply of ATP needed for the process of amino acid incorporation.

The result obtained with liver-tumour mitochondria is also included in Table 7, showing that the labelling of their protein by incubation with [^{14}C]leucine is similar to that of normal liver mitochondria, in contrast with the results obtained with microsomes.

DISCUSSION

It has been shown by Zamecnik, Keller, Littlefield, Hoagland & Loftfield (1956) that the incorporation of amino acid, which takes place when liver microsomes are incubated under the conditions used in the present experiments, involves peptide bonds. Since, however, it is not at present possible to demonstrate the net synthesis of a specific protein in such systems it may be doubted whether *de novo* protein synthesis is being studied. It has been demonstrated recently that in such a system the amino acids are incorporated into a substance having the immunological characteristics of rat serum albumin (Campbell, Greengard & Kernot, 1958). Such systems therefore offer the best prospects at present available of studying protein synthesis in subcellular particles.

One way of determining the extent to which amino acid incorporation in the disrupted cell is related to protein synthesis in the whole cell is to compare the amino acid incorporation under different metabolic conditions. For this purpose a comparison of normal and regenerating liver has much to commend it. Eliasson, Hammarsten, Reichard, Åqvist, Thorell & Ehrensvärd (1951) found that the incorporation of [^{15}N]glycine into the proteins of regenerating rat liver in the whole animal was highest 56 hr. after hepatectomy.

It has previously been shown that when slices of liver and regenerating liver are incubated with [^{14}C]glycine there is a much greater degree of incorporation of radioactivity into the proteins of the regenerating liver than the normal liver, but that the pattern of intracellular incorporation is similar in both tissues (Campbell *et al.* 1957). Since the concentration of amino acids in the regenerating liver is greater than that in the normal liver (Christensen, Rothwell, Sears & Streicher, 1948) this result is unlikely to be due to the difference in the radioactivity of the precursor amino acids. This difference between the ability of the microsomes present in the whole cells, as slices of tissue, is also shown when these microsomes are isolated and then incubated. In the present experiments the microsomes of regenerating liver showed approximately three times the ability of the microsomes from normal liver to incorporate amino acids. Thus it should be possible to use this subcellular preparation to investigate the factors underlying the difference between this fast-growing tissue and its normal counterpart. Such studies have been undertaken in greater detail by Hultin & von der Decken (1957) and von der Decken & Hultin (1958).

Such a parallelism between slices and subcellular preparations can, however, only occur if the process of cellular disruption damages the microsomes from the two types of tissue to the same extent. This may account for the failure to detect such a correlation when microsomes from normal liver are compared with those from liver tumour. Thus whereas liver-tumour microsomes in slices are somewhat more active than those of normal liver, they lose much of their activity when homogenized. In this connexion it is of interest that microsomes from the Ehrlich ascites tumour are less active than those from liver, although the reverse is true if the incorporation into intact cells is studied (Littlefield & Keller, 1957).

Zamecnik & Stephenson (1949) showed, by an enzymic method, that the concentration of glutathione in liver tumour was very low compared with that of the normal liver. This was confirmed by Campbell & Halliday (1957), who studied the composition of tissue extracts by ion-exchange chromatography. Sachs (1957), using a freeze-dried preparation of liver microsomes, showed that glutathione stimulated the incorporation of radioactive leucine. It seemed possible therefore that the low activity of the tumour microsomes could have been due to the low level of glutathione. That this is at least in part true is shown by the stimulation of amino acid incorporation when glutathione is added to tumour microsomes. The failure in the present experiments to obtain any effect on the incorporation into normal-liver microsomes by glutathione is possibly due to the use of a crude cell-sap fraction rather than the purified one used by Sachs. He also showed that there was an optimum concentration of glutathione, higher concentrations being inhibitory.

The nuclear fraction isolated from liver-tumour slices which were incubated with [^{14}C]glycine was much more active than that of normal liver (Campbell *et al.* 1957). In the present studies no difference between the nuclear fraction of the tumour and the liver could be detected. All three types of nuclear fraction inhibited the incorporation of amino acid into microsomes. Although the reason for this inhibition of the incorporation in one subcellular fraction by that of another is not understood at present, a practical point does emerge. If, when the whole homogenate of a tissue is incubated with a radioactive amino acid, the incorporation is very low, it is still possible that the incubation of

the individual subcellular fractions might produce more rewarding results.

In liver and in some other tissues, after the injection of radioactive amino acid, it is the microsomal protein which becomes highly labelled before the other fractions. It was suggested (Littlefield, Keller, Gross & Zamecnik, 1955) that this fraction is the primary site of protein synthesis in the cell. However, the possibility that organelles other than microsomes possess the ability to synthesize protein has not been excluded. Allfrey, Mirsky & Osawa (1957) have provided evidence that thymus nuclei are able to synthesize proteins without the contribution of other fractions. Bates, Craddock & Simpson (1958) have recently demonstrated the incorporation of [^{14}C]valine into cytochrome *c* by isolated rat-liver mitochondria, suggesting that mitochondria, also, are able to synthesize proteins. Although in the present studies the hepatic nuclei were inactive, the mitochondria had considerable activity. No attempt has been made to find the optimum conditions for the incorporation of amino acids into mitochondria, for the object was to study the effect of mitochondria on the incorporation into microsomes. The lowering of the microsome incorporation by mitochondria indicates that the two processes are not independent, but further studies have to decide whether a competition between the two synthesizing sites takes place or whether mitochondria exert a direct inhibition on microsomal incorporation. Elucidation of such problems may contribute to the understanding of the ways in which the characteristic subcellular distribution of amino acid incorporation, *in vivo* and *in vitro*, may be regulated.

SUMMARY

1. Microsomal preparations of regenerating liver incorporate three to four times as much [^{14}C]-glycine into their proteins as do those of normal liver, during a 50 min. incubation period.

2. Liver-tumour microsomal preparations have the ability to incorporate ^{14}C-labelled glycine, leucine and lysine into their proteins, in the presence of phosphoenolpyruvate. The extent of this incorporation, however, is much smaller than in preparations from normal liver.

3. Glutathione stimulates the incorporation of [^{14}C]lysine by preparations of liver-tumour microsomes.

4. The presence of the nuclear fraction from normal liver, liver tumour or regenerating liver greatly inhibits the incorporation of amino acids into the proteins of microsomal preparations.

5. Mitochondria of normal liver and of liver tumour are able to incorporate [^{14}C]leucine in the absence of microsomes, and this process is energy-dependent. When liver microsomes and mitochondria are incubated together, the incorporation into the microsomes is reduced and that into the mitochondria is increased.

6. The relevance of these studies, on the incorporation of amino acids into subcellular preparations, to the problem of the mechanism of protein synthesis, is discussed.

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A Comparison of the Serum Proteins of Normal Rats with those of Rats bearing Liver Tumours

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The persistent demand for a reliable method for the diagnosis of the presence of malignant tumours has led to numerous investigations on the composition of the serum proteins of animals bearing such tumours. So far, with the exception of myeloma in man, the serum proteins of the tumour-bearing animal have not been shown to differ from those of animals under other pathological conditions in any characteristic way. (Studies on the plasma proteins of patients with cancer were reviewed by Winzler in 1953.) In spite of this discouraging situation it was felt that yet another effort to investigate the problem by recently introduced techniques and discriminating immunological methods might be of value. The proteins in the serum of rats bearing well-characterized primary liver tumours induced by feeding 4-dimethylaminoazobenzene have therefore been compared with the serum proteins of the normal rat. While this work was in progress Darcy (1957) published a paper in which the composition of the serum proteins of normal and tumour-bearing rats was compared by immunological methods. The results now presented confirm and extend these studies.

A preliminary report of some aspects of this work has appeared (Campbell, Kernot & Roitt, 1957).

MATERIALS AND METHODS

Animals. Rats were male Wistar albinos highly inbred in this Institute. Liver tumours were induced by feeding the rats on a diet containing 0·06% of 4-dimethylaminoazobenzene as described by Campbell (1955). Partial hepatectomy in rats was performed by the method of Higgins & Anderson (1931).

Rabbit antisera. An alum-precipitate of freshly obtained rat serum was prepared by the method of Proom (1943). Rabbits were given this suspension by intravenous injection as previously described (Campbell, 1955). The antiserum obtained was not fractionated before use, but was stored at −20°.

Precipitation in agar gel. This was carried out by the method of Ouchterlony (1953). Sodium azide (1%) was incorporated into the medium as a bacteriocide. The wells were not replenished. The plates were photographed by the method of Lawson (1957). When it was necessary to compare the antigenic composition of the different rat sera, some normal serum was included in the agar used for the plate. This follows the method of Björklund (1952, 1953).

Immunoelectrophoresis. This was carried out in agar by a slight modification of the method of Grabar & Williams (1955).

Fractionation of plasma proteins

Salt. When $(NH_4)_2SO_4$ was used an equal volume of a saturated solution was added to rat serum obtained by heart puncture. The precipitate was removed by centrifuging and was washed with a solution of half-saturated $(NH_4)_2SO_4$. The precipitate was dissolved in water, dialysed and freeze-dried. The protein in the supernatant from the $(NH_4)_2SO_4$ fractionation was recovered in a similar manner.

When Na_2SO_4 was used for fractionation 2 vol. of 27% (w/v) Na_2SO_4 dissolved in potassium phosphate buffer (pH 7·8, *I* 0·1) was added to 1 vol. of rat serum, to give a final concentration of 18% (w/v) Na_2SO_4. The precipitate and supernatant were treated as described for $(NH_4)_2SO_4$ fractionation.

Paper electrophoresis. The fractionation was followed by electrophoresis on Whatman no. 3 MM paper, with veronal buffer (pH 8·6, *I* 0·05) as described by Flynn & de Mayo (1951). In general, this buffer gave much better results than did that containing acetate described by Roberts & Kelley (1956). More recently, satisfactory results have been obtained with the 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–ethylenediaminetetra-acetic acid (EDTA) buffer of Aronsson & Grönwall (1957). The method of Kohn (1957), which involves the use of cellulose acetate paper with veronal buffer, was also employed.

Zone electrophoresis. The method of Porath (1956) in which electrophoresis is carried out in a column was used. The columns were packed with cellulose prepared as described by Flodin & Kupke (1956) in a borate–phosphate buffer (0·0115 M-$Na_2B_4O_7$–0·0155 M-NaH_2PO_4, pH 8·6, *I* 0·05).

RESULTS AND DISCUSSION

Paper electrophoresis

The serum proteins of the rat do not give discrete bands when subjected to paper electrophoresis in veronal buffer as do those of man and rabbit. The results obtained in the present experiments were, in general, similar to those obtained by Roberts & Kelley (1956), although the buffer used by these authors was not found satisfactory. No system of paper electrophoresis has so far been found which gives completely satisfactory results for rat serum. Thus the best overall picture is obtained on Whatman no. 3 MM paper with veronal buffer. Under these conditions the α_1-globulin is only

just separated from the albumin (Fig. 1*a*), as may be more clearly shown when the proteins precipitated after half-saturation of the serum with ammonium sulphate are subjected to electrophoresis (Fig. 1*b*). If a clearer separation between these two components is required the method of Kohn (1957) with cellulose acetate paper and veronal buffer is to be recommended (Fig. 1*d*). An excellent separation of the α_1-, α_2- and β-globulins may be obtained by the use of the tris–EDTA buffer of Aronsson & Grönwall (1957). However, in this case the front of the albumin band is rather diffuse (Fig. 1*c*).

The paper-electrophoretic method of Aronsson & Grönwall (1957) revealed a difference between the proteins in the α_1-, α_2-globulin band of the normal and tumour-bearing rat serum. The latter appeared to contain additional components compared with that of the normal serum.

Salt fractionation

Paper electrophoresis of the two fractions obtained by half-saturation with ammonium sulphate showed that both contained albumin, although the amount in the precipitate was very small compared with that in the supernatant. The fractionation of the γ-globulin was complete in that it was present only in the precipitate. There was no satisfactory fractionation of the α_1-, α_2- and β-globulins by this procedure.

The use of sodium sulphate to fractionate the sera has the advantage that there is a complete separation of both albumin and γ-globulin. Thus the albumin was confined to the supernatant and the γ-globulin to the precipitate. The other components were distributed between the two fractions.

Zone electrophoresis

The result of submitting normal serum to zone electrophoresis after removal of the precipitate obtained by half-saturation with ammonium sulphate is shown in Fig. 2. No consistent difference between the results obtained with this serum fraction from normal and tumour-bearing animals has been obtained. However, when the precipitate obtained by half-saturation of normal serum with ammonium sulphate was submitted to zone electrophoresis the pattern shown in Fig. 3 was obtained, whereas electrophoresis of a similar fraction from tumour-bearing animals gave the somewhat different pattern shown in Fig. 4. The difference between the central parts of these two patterns has been a consistent finding and suggests

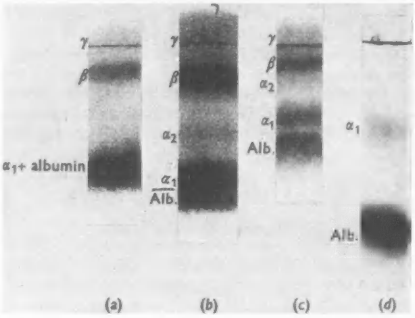

Fig. 1. Paper electrophoresis of rat serum proteins under various conditions. (*a*) Whole serum, Whatman no. 3MM paper, veronal buffer. (*b*) Precipitate obtained by half-saturating the serum with $(NH_4)_2SO_4$. Whatman no. 3MM paper, veronal buffer. (*c*) Whole serum, Whatman no. 3MM paper, tris–EDTA buffer. (*d*) Impure albumin obtained by zone electrophoresis on cellulose columns (Campbell & Stone, 1957). Cellulose acetate paper, veronal buffer.

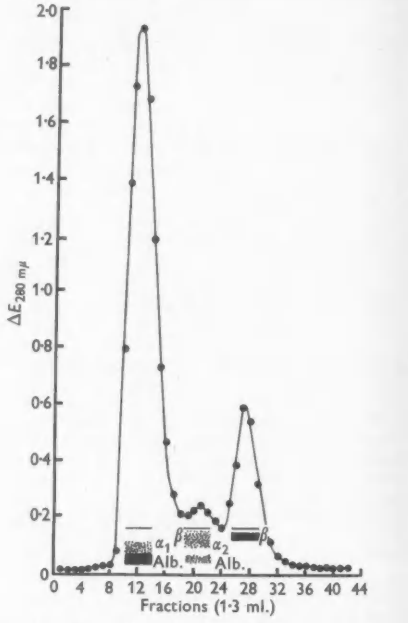

Fig. 2. Zone electrophoresis of normal-rat serum proteins after removal of the precipitate formed by half-saturation with $(NH_4)_2SO_4$. Protein (50 mg.) was added to the column (46 cm. × 1·5 cm.) packed with 'cellulose'. A current of 10 mA (500 V) was applied for 24 hr. An indication of the composition of the peaks is given diagrammatically. The diagrams represent the results obtained by paper electrophoresis in veronal buffer.

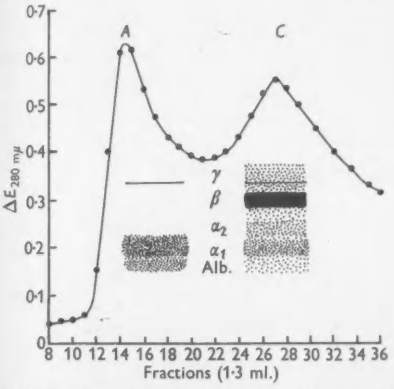

Fig. 3. Zone electrophoresis of normal-rat serum proteins precipitated from whole serum by half-saturation with $(NH_4)_2SO_4$. Conditions were as described for Fig. 2.

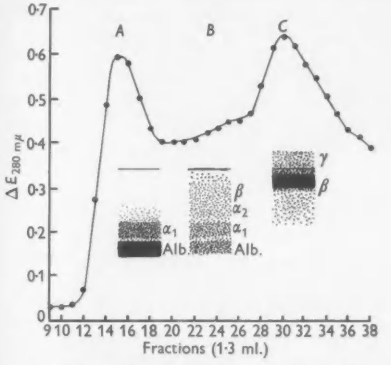

Fig. 4. Zone electrophoresis of serum proteins of tumour-bearing rat precipitated from whole serum by half-saturation with $(NH_4)_2SO_4$. Conditions were as described for Fig. 2.

an increase in some component in the tumour serum compared with the normal having the properties of an α-globulin.

Differences between the sera revealed by immunological techniques

When the Ouchterlony technique of diffusion of antibody and antigen in an agar gel was applied to serum from normal and tumour-bearing rats and their corresponding antisera, more lines were revealed in the serum from tumour-bearing rats than from the normal serum. For this reason in all subsequent experiments antiserum to the tumour serum was used. A typical result is shown in Plate 3 (*a*). The difference may be more clearly defined by absorbing the antibodies to normal serum according to the Björklund (1953) technique. If a similar plate is set up differing only in that the agar contains some normal serum, then, as shown in Plate 3 (*b*), the tumour serum shows an additional line compared with the normal. It may be observed that the pattern of the precipitated proteins around the centre well is distorted by the advancing antigen.

If instead of mixing the normal serum with the agar the normal serum is placed in the centre well together with the antiserum the result shown in Plate 3 (*c*) is obtained. It will be observed that here three extra lines are present in the tumour serum. When the amount of normal serum in the centre well is reduced a line does appear with normal serum and this line is confluent with one of the lines in the tumour serum. This suggests that at least one of the components is merely present in the tumour serum at an increased concentration compared with the normal. Further evidence on this point will be presented later.

Owing to the simplicity of the Björklund technique further characterization of the additional antigens in the tumour serum was made by this method. When instead of fresh serum the serum used was freeze-dried and then dissolved in 0·9% sodium chloride (1·5 mg. of protein/0·2 ml. of saline), the tumour serum again gave the additional line, showing that the responsible antigen withstood freeze-drying and was probably not a lipoprotein. Ammonium sulphate appears to effect a fractionation of the component, for the supernatant failed to give a line whereas the precipitate gave a line when tested at a concentration of 1·5 mg. of protein/0·2 ml. of saline. On the other hand, the use of sodium sulphate did not effect a fractionation of the component, both the precipitate and the supernatant giving the additional line.

To obtain further information about the nature of the additional components in tumour serum the fractions obtained after zone electrophoresis of the ammonium sulphate precipitate were tested. All three fractions (see Fig. 4) gave a line, but, as judged by the distance of the line from the centre well, time of appearance and bulk of precipitate, fraction *B* contained more of the antigen than did either fraction *A* or fraction *C*. This experiment suggested that additional components in the tumour-serum proteins had the mobility of an α-globulin. Further evidence on this point was obtained by immunoelectrophoresis. The results of this experiment are shown in Fig. 5. It can be seen that the tumour serum contained two additional components having the mobility of α_1- and α_2-globulins respectively in the buffer of Aronsson

& Grönwall (1957). A control experiment in which the normal serum replaced the tumour serum failed to reveal any lines.

The specificity of the unknown components with respect to tumour serum was studied as follows. Tests were made on the fractions obtained after zone electrophoresis of the proteins of normal serum precipitated by half-saturation with ammonium sulphate (fractions *A* and *C*, Fig. 3). These fractions both showed the presence of the component. Thus it is clear that the component(s) is present in normal serum and is merely increased in tumour serum. Serum obtained from a rat 40 hr. after a partial hepatectomy was then tested, together with that from a pregnant rat. In each a line appeared at about the same time as with the tumour serum and all three lines merged. Thus not only is there an increase in the concentration of the component in tumour serum but also in other conditions involving rapid growth.

An attempt was made to determine whether all or any of the components were glycoproteins, by staining the Ouchterlony agar plate with the periodic acid–Schiff's reagent according to the method of Uriel & Grabar (1956). The results obtained were consistent with the view that at least one of the components was a glycoprotein.

The increase in the concentration of the proteins comprising the α-globulin fraction of the plasma in the rats bearing liver tumours compared with normal rats is in agreement with the work of Bernfeld & Homburger (1955), who studied the influence of tumour growth on the plasma proteins in mice and later (Nisselbaum & Bernfeld, 1956) isolated glycoproteins from the plasma of both normal and tumour-bearing mice. The results of the present experiments are in close accord with those of Darcy (1957) already mentioned. The Björklund technique used with Ouchterlony agar plates has, however, greatly simplified the detection of the proteins, the concentration of which is increased in the tumour-bearing animal. Zone electrophoresis has provided confirmatory evidence of the nature of the substances and immunoelectrophoresis has shown that the concentration of at least two plasma proteins having different electrophoretic mobilities is increased in the tumour-bearing rats.

Although these experiments do not provide any evidence for a difference between normal serum and the serum from tumour-bearing animals which is confined to such animals, they do indicate a change in the serum proteins which arises as a result of growth. Bucher, Scott & Aub (1951) have shown that substances in the blood can initiate mitosis. Thus when one partner of parabiotic twins was subjected to partial hepatectomy the rate of mitosis of the unoperated animal rose. Whether the components studied in the present investigation are responsible for controlling the growth of tissues or whether they are produced as a result of growth is not known.

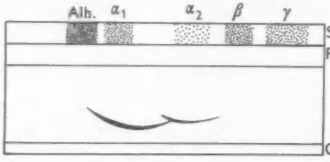

Fig. 5. Immunoelectrophoresis of serum proteins from a tumour-bearing rat. The proteins were first separated by electrophoresis on Whatman no. 3MM paper with tris–EDTA buffer. The paper was cut longitudinally, one half being stained and the other placed on a sheet of agar as indicated. The separation of the proteins into bands as revealed by the stained strip is also indicated. A channel was cut in the agar and this was filled with antiserum to the serum proteins of the tumour-bearing rat absorbed with one-fifth of its volume of serum from a normal rat (cf. Plate 3c). The two lines on the diagram are tracings of a photograph taken after 10 days.

SUMMARY

1. The composition of the serum proteins of normal rats and those bearing liver tumours has been compared by paper electrophoresis, zone electrophoresis and immunological methods.

2. The electrophoretic methods, particularly zone electrophoresis, showed that the serum protein of the tumour-bearing animals contained more protein of the mobility of the α-globulins than did normal serum.

3. Immunological methods, particularly the Ouchterlony plate method as modified by Björklund, showed that one or more components of the normal serum proteins were present in increased concentration in the tumour serum. Similar results were obtained with serum from either pregnant rats or rats after partial hepatectomy.

4. Immunoelectrophoretic methods showed that the proteins present in an increased concentration in the tumour serum had mobilities corresponding to α_1- and α_2-globulins respectively.

We would like to thank Professor F. Dickens, F.R.S., for his encouragement and the British Empire Cancer Campaign for a grant to the Medical School.

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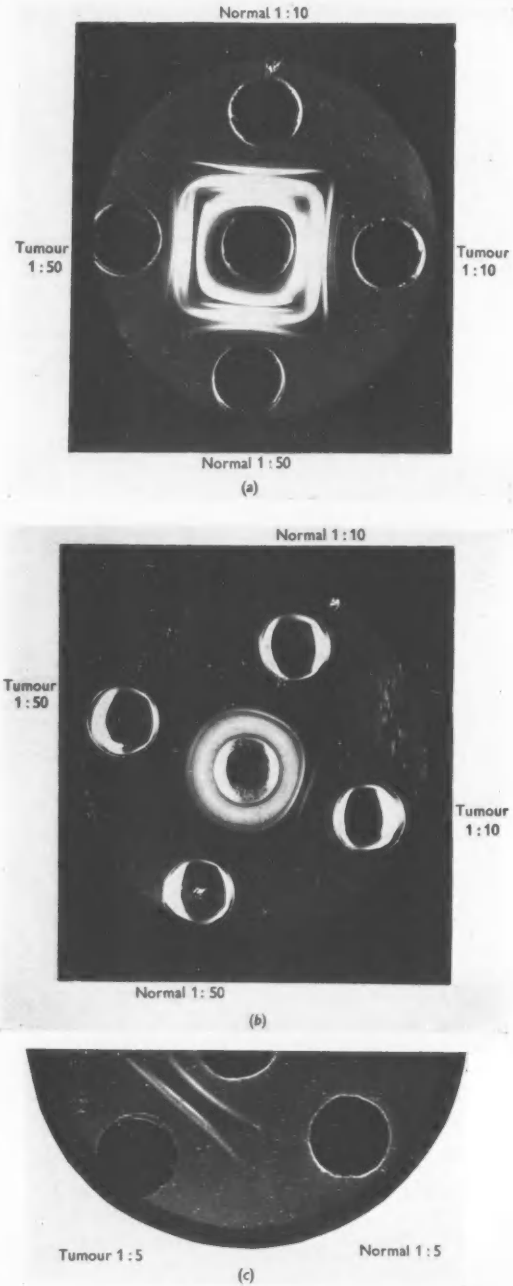

P. N. CAMPBELL, BARBARA A. KERNOT AND I. M. ROITT—A COMPARISON OF THE SERUM PROTEINS OF NORMAL RATS WITH THOSE OF RATS BEARING LIVER TUMOURS

(*Facing p.* 159)

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EXPLANATION OF PLATE 3

(*a*) Ouchterlony plate in which an antiserum to the serum proteins of a tumour-bearing rat was placed in the centre well. The other wells contained either whole serum from normal rats or serum from tumour-bearing rats diluted with saline as indicated. Photographed after 12 days.

(*b*) Ouchterlony plate set up exactly as described in (*a*) except that the agar contained 1% (v/v) of normal-rat serum.

(*c*) Ouchterlony plate in which the centre well contained antiserum to tumour serum absorbed with one-fifth of its volume of normal-rat serum. The side wells contained either tumour serum or normal serum diluted with saline as indicated. Photographed after 10 days.

Mitochondria from Embryonic Tissues of the Chick

1. PREPARATION, CHARACTERIZATION AND SOME ENZYMIC PROPERTIES

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(*Received* 14 *May* 1958)

Whereas the isolation and properties of subcellular particles from normal adult tissues (review: Hogeboom & Schneider, 1955), tumour tissue (Schneider, 1946), plant tissues (review: Hackett, 1955) and yeast (Linnane & Still, 1955) have been extensively studied, no comparable work seems to have been done with embryonic tissues. Claude (1938, 1940) centrifuged particles from a homogenate of previously frozen chick-embryo pulp in phosphate buffer; they contained some ribonucleic but no deoxyribonucleic acid. Steinbach & Moog (1945) and Moog & Steinbach (1946) obtained granules from homogenates of chick embryos in water by centrifuging at room temperature, and studied their phosphatase and adenosine triphosphatase activities.

Recknagel (1950) homogenized eggs of the frog *Rana pipiens* in phosphate buffer and separated a granular fraction which contained about 70% of the cytochrome oxidase activity of the homogenate, with a much higher specific activity, and which stained with Janus green B. A similar mitochondrial fraction, in which the cytochrome and succinate oxidases were localized, was obtained by Boell & Weber (1955) from homogenates in sucrose solution of embryos of the toad *Xenopus laevis*.

Nuclear, mitochondrial and microsomal fractions have been obtained by differential centrifuging of homogenates in sodium chloride–phosphate medium of embryos of the grasshopper *Melanoplus differentialis* (Bodine & Lu, 1950, 1952); all the succinate oxidase activity was in the mitochondrial fraction.

Hutchens, Kopac & Krahl (1942) separated from homogenates of eggs of the sea urchin *Arbacia punctulata* a 'mitochondrial' fraction which, however, contained only 1% of the cytochrome oxidase activity. On the other hand, Weber is reported by Boell & Weber (1955) to have found that homogenates of eggs of *Paracentrotus lividus* yield large granules which sediment between 600 and 12 000 *g*, have the characteristics of mitochondria and contain 90% of the cytochrome oxidase activity of the homogenate.

Clearly, cell-fractionation studies with embryonic tissue have so far been scanty and incomplete. Attempts have been made to separate a fraction containing enzymes that are associated with mitochondria in adult mammalian tissues, but in few cases have the workers carried out the fractionation in sucrose medium which, like certain special salt

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solutions (Chappell & Perry, 1954), allows the preparation of satisfactory particles. In particular, hypotonic and phosphate-containing media are likely to cause severe damage to the mitochondria (see, for example, Dituri, Shaw, Warms & Gurin, 1957).

The work described in this and the accompanying paper (Carey & Greville, 1959) represents an attempt to apply modern biochemical criteria to the problem of the nature and enzymic constitution of subcellular particles in embryonic tissue. Chick embryos were chosen, as they were available throughout the year. They were used whole after incubation for 5 days. In earlier stages, the amount obtainable would be very small, and in later stages, owing to considerable differentiation, the tissues might become more characteristic of the adult state.

EXPERIMENTAL

Abbreviations

ATP, Adenosine triphosphate; CoA, coenzyme A; DNA, deoxyribonucleic acid; DNP, 2:4-dinitrophenol; EDTA, ethylenediaminetetra-acetic acid; RNA, ribonucleic acid; tris, 2-amino-2-hydroxymethylpropane-1:3-diol.

Materials

Water distilled in glass was used and, when possible, AnalaR reagents. Acidic substances were neutralized with NaOH before addition to media.

The specimen of CoA, obtained some years previously from Pabst Laboratories, Milwaukee, Wis., U.S.A., was found by the method of Kaplan & Lipmann (1948) to have an activity of 208 units/mg. (approx. 50% purity).

Cytochrome *c*, prepared by the method of Keilin & Hartree (1945), was purified by adsorption on the ion-exchange resin IRC 50 (Margoliash, 1954).

Tris (L. Light and Co. Ltd.) was recrystallized from ethanol; 'Sigma 121' (tris supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A., for use as a primary volumetric standard) was used without further purification.

EDTA (L. Light and Co. Ltd.) was recrystallized from water.

Commercial DNP was recrystallized once from ethanol and twice from aq. ethanol; m.p. 112–113°.

The crystalline disodium salt of ATP was obtained from the Sigma Chemical Co., and glutathione from The Distillers Co. Ltd.

Preparative methods

Production of embryos. The eggs, all Rhode-Island Red × Light Sussex, were supplied by Withers and Son, Appelby Farm, Egerton, Ashford, Kent, who collected them within 4 hr. of laying. They were incubated at 38–39°, and the stage of development of embryos was determined by comparison with illustrations in Lillie's *The Embryology of the Chick* (Hamilton, 1952). Here, '5-day embryo' refers to embryos in stages 25–27, the majority being at stage 26. The stages of later embryos were not so accurately determined.

Separation of cell fractions. (*a*) Whole embryos: about eight embryos from approximately 12 eggs were collected in 0·31 M-sucrose or 0·15 M-KCl at room temperature and, after freeing from extra-embryonic membranes and yolk, transferred to ice-cold preparation medium (see below). These operations were completed in 5–8 min. Subsequent manipulations were carried out at 0–2°. The embryos (1·2–1·5 g.) were homogenized in 10 vol. of fresh medium by brief use (about 30 sec.), at low speed (300–500 rev./min.), of a Potter–Elvehjem device with Perspex rotor. Differential centrifuging was then carried out as shown in Table 1, which also gives the symbols assigned to the various fractions (cf. de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). When required for enzymic studies, fraction *M* was washed twice or thrice on the centrifuge, each time with a volume of medium equal to five to eight times the total volume of the embryos and centrifuging for the same time and with the same centrifugal force as in the original sedimentation of the mitochondria. (*b*) Liver tissue: this was treated similarly, except that material from hatched chicks was finely cut with scissors before homogenization.

Extracts from acetone-dried powders. Washed mitochondria from about 30 g. of liver of 12- to 14-day chicks, or from 6 to 8 g. of liver of 16-day embryos, were prepared with sucrose medium III (see below) and suspended in 5–10 ml. of the same medium at 0°. Acetone at −5° (20 vol.) was added, and the ppt. centrifuged at −5° and resuspended in acetone at −5°. This was repeated twice, and the powder (20–30 mg./g. of liver) dried at 0·1 mm. Hg (approx.) over P_2O_5, NaOH and paraffin wax, and stored in a desiccator below 0°. Clear extracts containing 'acetate-activating enzyme' were obtained by treating the powder for 20 min. at 0° with 5 mM-tris chloride buffer, pH 7·5 (1 ml./3–20 mg. of powder), and centrifuging at 12 000 *g* for 15 min.

Preparation media

The various media used in the preparation of cell fractions were as follows.

Salt medium. 0·10 M-KCl; 0·05 M-tris chloride buffer, pH 7·5; mM-EDTA; mM-ATP; 5 mM-$MgSO_4$.

Table 1. *Fractionation of chick-embryo homogenate*

Supernatants from the first and second centrifugings are designated S_N and S_M respectively. For description of media see text.

Salt medium		Sucrose media			
Force (*g*)	Time (min.)	Force (*g*)	Time (min.)	Fraction	Symbol
600	5	600	15	Nuclei + unbroken cells	*N*
8 500	10	12 000	10	Mitochondria	*M*
105 000	60	105 000	60	Microsomes	*P*
—	—	—	—	Final supernatant	S_P

Sucrose I. 0·20 M-sucrose; 0·05 M-tris chloride buffer, pH 7·5; mM-EDTA; mM-ATP; 5 mM-$MgSO_4$.

Sucrose II. 0·29 M-sucrose; mM-EDTA; mM-ATP; 5 mM-$MgSO_4$.

Sucrose III. 0·31 M-sucrose; mM-EDTA.

Sucrose IV. 0·31 M-sucrose.

The salt medium was that found by Chappell & Perry (1954) to be most suitable for the preparation of pigeon-breast-muscle mitochondria, and might be expected to be applicable to the avian tissue used in the present work. Addition of EDTA to sucrose media used for preparation of muscle mitochondria was introduced by Slater & Cleland (1952). We find that fractionation of chick-embryo homogenates is much easier in salt solution and sucrose media I and II than in 0·31 M-sucrose, since the particles do not adhere so tenaciously to glass and plastic surfaces as they do in the last-named medium and so are resuspended more easily.

Analytical methods and enzyme assays

Total nitrogen. This was determined by a micro-Kjeldahl method with incineration for 12 hr. Mitochondrial N was obtained by subtracting from the N content of the mitochondrial suspension that of the medium in which the mitochondria were suspended. A similar procedure was used for the other fractions.

Nucleic acids. These were determined by the Schmidt–Thannhauser procedure (Volkin & Cohn, 1954), except that their hydrolysis products were separated by paper chromatography. The following procedure was recommended by Dr Roy Markham, F.R.S., for whose advice and guidance we are very grateful. After removal of the acid-soluble compounds and lipids from the sample, the residue was treated with N-KOH to hydrolyse the RNA. The solution was then placed in portions on to Whatman no. 3 MM paper so as to form a spot; after each addition an excess of 10 N-acetic acid was put round the periphery of the spot and the damp area dried with a jet of air; and after all additions had been made the spot was dried until no smell of acetic acid remained. The chromatogram was run with aq. 70% (v/v) propan-2-ol in an atmosphere of NH_3. Mononucleotides from the RNA were located by photography with transmitted u.v. light. The two spots so detected, which between them contain all four monoribonucleotides, were cut out and the material from each was eluted separately with 5 ml. of 0·1 N-HCl. The extinctions of the two solutions were measured at 260 mμ in a Beckman model DU spectrophotometer against blanks from the paper, and their sum was used as a measure of the RNA. The DNA, which remained at the starting line of the chromatogram, was hydrolysed by treatment with conc. HCl, which was allowed to dry at room temperature. A chromatogram was then run with butanol (saturated with water)–formic acid (9:1, v/v). The adenine liberated from the DNA by the treatment with HCl was located as above and eluted with 5 ml. of 0·1 N-HCl, and the extinction at 260 mμ was used as a measure of the DNA.

Succinate oxidase. This was assayed manometrically at 37° by the procedure of Schneider & Potter (1943), activity in the absence of added succinate being subtracted.

Adenosine triphosphatase. This was determined by measuring the inorganic phosphate released in 10 min. at 23° by 0·1 ml. of a mitochondrial suspension in a medium (total vol., 1 ml.) containing 0·05 M-tris chloride buffer, pH 7·5, and 2 mM-ATP, and brought to 0·31 osmolar, with sucrose when the mitochondria were prepared in sucrose-containing media, or with KCl when they were prepared in salt medium. The reaction mixture was deproteinized by addition of 1·8 ml. of 4·5% (w/v) trichloroacetic acid and filtration through Whatman no. 42 paper. Phosphate was determined in a 2 ml. portion by the method of Fiske & Subbarow (1925) with a Beckman model DU spectrophotometer at 650 mμ.

RESULTS

Characterization of cell fractions from 5-day embryos

Microscopic and staining studies. Under the phase-contrast microscope fraction *N* was seen to consist of debris and unbroken cells, small particles showing Brownian movement, and somewhat larger bodies which predominated and were identified as nuclei, since they could be stained by the Feulgen procedure. Small lighter areas within the stained nuclei presumably represented nucleoli. When prepared in 0·31 M-sucrose the nuclei were 10–15 μ in diameter, but with ATP and Mg^{2+} ion in the medium only 4–5 μ. With lower concentrations of sucrose they swelled and frequently burst.

Fraction *M* under the phase-contrast microscope (×1425) appeared to consist almost entirely of refractile particles which were just visible and exhibited Brownian movement. They were stained blue by Janus green B. If an oxidizable substrate (succinate) were added and oxygen excluded, the stained particles became purple in 6 hr. and red in 24 hr. Such behaviour, however, does not identify them for certain as mitochondria, since separated cell particles other than mitochondria may take up and reduce this dye (Lazarow & Cooperstein, 1953).

Fraction *P* was difficult to resuspend, but the agglutinated masses seen under the microscope in no way resembled agglutinated mitochondria. The centrifuged *P* pellet was bluish and hyaline, whereas the *M* pellet was mushroom-coloured and opaque. Paigen (1956) has observed that mouse-liver microsomes adsorb haemoglobin. When haemoglobin was added to chick-embryo homogenate, the *P* pellet when isolated was bright red whereas no change was seen in the *M* pellet.

S_P appeared completely clear under the phase-contrast microscope.

To provide further evidence for the identification of the various cell fractions, the distributions between them of total nitrogen, RNA, DNA and succinate oxidase were determined for comparison with the findings with adult-rat liver.

Distribution of total nitrogen, ribonucleic acid and deoxyribonucleic acid. (Fig. 1). The distribution in cell fractions prepared both in salt medium and in sucrose medium I are seen to be similar to those found with adult-rat liver fractionated in sucrose solution (Schneider, 1948), except that (i) fraction *N* contained a higher proportion of the total

nitrogen in the embryo preparations than it did in those from adult-rat liver, and (ii) up to 4 % of the DNA was found in fraction *M* of embryo preparations. The DNA in *M* could be reduced to nothing if fraction *N* were spun down harder, but as the main object was to prepare mitochondria in good yield as rapidly as possible, the slight nuclear contamination was accepted.

Brachet & Jeener (1943–45) showed that, whereas in adult tissues most of the RNA was to be found in particles sedimenting at accelerations up to 75 000 *g*, with frog oocytes a large proportion remained in the supernatant. The latter is not true of chick-embryo homogenates, in which most of the RNA is particulate.

Proportion of the succinate oxidase activity in the mitochondrial fraction. (Table 2). Fractions *N* and S_M were combined before the activity was measured, preliminary work having established that the activity in the combined fractions was equal to the sum of their separate activities. About 70 % of the activity of the homogenate was found in *M*, and about 25 % in the remaining fractions. Activity/mg. of nitrogen was many times greater in *M* than in the homogenate. Activity in fraction *N* was presumably due to unbroken tissue and mitochondria; on washing *N*, activity passed from this fraction into the wash fluid.

The above distribution and staining studies indicate that cell fractions similar to those obtained from adult tissues can be prepared from 5-day chick embryos.

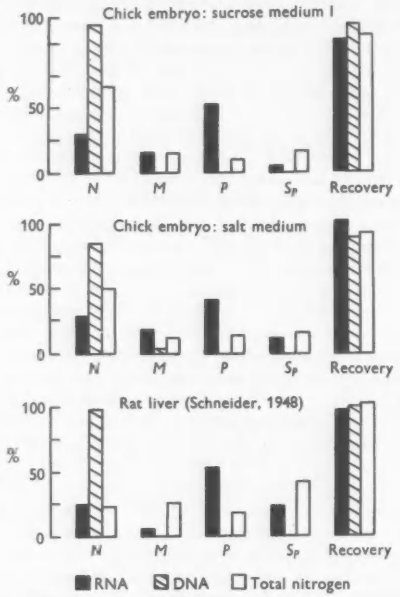

Fig. 1. Distribution of RNA, DNA and total nitrogen in cell fractions of chick embryo and rat liver. The RNA in each fraction is expressed as a percentage of the RNA in the homogenate, and 'recovery' is the sum of the percentage values of all the fractions. DNA and total nitrogen are treated similarly.

Adenosine triphosphatase activity of mitochondria from 5-day embryos

It is well known (e.g. Kielley & Kielley, 1951; Lardy & Wellman, 1953) that rat-liver mitochondria, when freshly isolated by suitable techniques, have a low adenosine triphosphatase activity, which is greatly stimulated by DNP. If the particles are aged or subjected to treatment considered to be damaging, the adenosine triphosphatase activity in the absence of DNP increases, whereas that in the presence of DNP is not so great as it is with fresh, untreated mitochondria. The activities in the presence and absence of DNP may thus give some indication whether mitochondria have undergone serious deterioration as a result of an unsuitable preparation technique. We have applied these tests in an attempt to assess the condition of our mitochondria prepared in the various media (Table 3). It is seen that preparation in different media led to different enzymic activities, whereas the percentage increases in activity in the presence of DNP were all of the same order.

Table 2. *Proportion of the succinate oxidase activity in the mitochondrial fraction*

Chick embryo was fractionated in sucrose medium I. Values for rat liver are derived from the data of Hogeboom, Schneider & Pallade (1948).

	Chick embryo			Adult-rat liver		
		Succinate oxidase			Succinate oxidase	
Fraction	Nitrogen (%)	(%)	(μl. of O_2/mg. of N/hr.)	Nitrogen (%)	(%)	(μl. of O_2/mg. of N/hr.)
Homogenate	[100]	[100]	188	[100]	[100]	770
M	10·2	70·2	1273	26·0	71·8	2120
$N+S_M$	84·4	25·4	57	72·9	20·4	214
Sum of *M* and ($N+S_M$)	94·6	95·6	—	98·9	92·2	—

Table 3. *Adenosine triphosphatase activity of mitochondria from 5-day chick embryos in the presence and absence of 2:4-dinitrophenol*

For description of technique and media see Experimental section. Activities are given as μmoles of phosphate released/mg. of N/hr., means and standard errors being shown. In the columns headed % the activity in the presence of DNP is given as a percentage of that in its absence.

Preparation medium	No. of preparations	DNP absent (activity)	0·01 mM-DNP		0·1 mM-DNP	
			Activity	%	Activity	%
Salt	4	16·4±2·4	26·0±3·3	159	51·7±5·5	315
Sucrose I	2	18·8±0·15	26·7±3·0	142	42·3±3·7	225
Sucrose II	5	6·5±0·4	10·5±0·6	162	16·8±1·7	258
Sucrose IV	2	3·4±0·3	6·1±0·6	179	11·8±2·6	347

It is clear that activity in the absence of DNP was much higher with mitochondria prepared in salt medium and sucrose medium I, both of which contained tris, than in sucrose medium II, which did not. Again, it was found that mitochondria prepared in salt medium showed a higher rate of oxygen uptake/mg. of mitochondrial nitrogen with various substrates than those prepared in sucrose medium II (Table 1 of the next paper, Carey & Greville, 1959). The possibility had to be considered that the presence of tris in the medium was causing an error in the determination of mitochondrial nitrogen, giving a low value with consequent falsely high specific activity. It might then be expected that the yield of mitochondria, measured as nitrogen, would be lower in media containing tris than in those not. However, it was found that the average yield of mitochondria, in mg. of nitrogen/embryo, was 0·065 with media containing tris (25 preparations) and 0·064 with media free from tris (20 preparations). The possibility of error due to tris was tested more directly as follows: various volumes of a suspension of embryo mitochondria in 0·31 M-sucrose were diluted to 0·5 ml. with 0·31 M-sucrose, and 0·5 ml. of a solution containing 0·14 M-sucrose and 0·1 M-tris, pH 7·5, was added. The concentration of tris in these diluted suspensions was then the same as in salt medium and sucrose medium I. The nitrogen contents of the various suspensions, and of the tris solution diluted with an equal volume of 0·31 M-sucrose, were then determined. The mitochondrial-nitrogen content of each diluted suspension was calculated (*a*) from the difference between the nitrogen contents of diluted suspension and diluted tris solution, and (*b*) from the nitrogen content of the undiluted suspension. (*a*) was never less than (*b*), so it was concluded that the presence of tris did not lead to low values for mitochondrial nitrogen.

Cytochromes in mitochondria from 5-day embryos

It has been reported (Potter & DuBois, 1942; Yaoi, 1928–29) that cytochrome *c* cannot be detected in chick embryos at the stage used in the present study. The observation in the next paper (Carey & Greville, 1959) that the respiration of mitochondria from 5-day chick embryos is inhibited by cyanide suggests, on the other hand, that the hydrogen transport is mediated by the cytochrome chain as in other tissues. It was therefore worthwhile to examine our mitochondria spectroscopically for the presence of cytochromes. We thank Dr R. Hill, F.R.S., for permission to use his apparatus for this purpose, and Dr D. S. Bendall for guidance. Table 4 shows that the mitochondria contain cytochromes similar to those in liver mitochondria. Of the bands observed, the cytochrome *b* bands of the embryo were least like those of liver. With the embryonic material at room temperature, in addition to the band at 605 mμ bands were spread over the region 550–568, with peaks at 565 and 552 mμ. The latter peak probably corresponds to cytochrome c_1, the cytochrome *c* band being too faint to observe clearly, although at liquid-air temperatures it was clearly distinguishable, albeit relatively weak. Possibly some of the cytochrome *c* was lost from the particles when they were thawed after storage, and was discarded with the supernatant when they were centrifuged for the

Table 4. *Cytochromes in mitochondria from 5-day chick embryos*

A suspension of mitochondria from 50 embryos was stored frozen. After thawing it was centrifuged (12 000 *g*, 15 min.) and $Na_2S_2O_4$ was added to the pellet. The spectrum was observed at room temperature with a Zeiss microspectroscope ocular (column i). The pellet was then suspended in sucrose medium II (1 ml.) and, after addition of glycerol (1 ml.) and a trace of $Na_2S_2O_4$, cooled in liquid air. The temperature was allowed to rise until devitrification occurred (Keilin & Hartree, 1949) and the spectrum was again observed (column ii). Values in parentheses are those given for liver mitochondria by Chance & Williams (1956).

Component	α-Band (mμ)	
	(i)	(ii)
a	605 (604–605)	603 (601)
b	565 (562–564)	563 (560)
c_1	552 (553–554)	554 (554)
c	— (550)	549 (549)

observations. However, despite the uncertainty about the amount of cytochrome *c* relative to that of the other cytochromes, there seems no reason to doubt that the hydrogen transport in chick-embryo mitochondria proceeds by the usual cytochrome route.

'*Acetate-activating enzyme*' *in liver from chick embryos and recently hatched chicks*

In connexion with the study of fatty acid oxidation by mitochondria from embryonic tissue described in the next paper (Carey & Greville, 1959) it became of interest to examine these particles for the presence of the 'acetate-activating enzyme'. This enzyme, also called 'acetyl-coenzyme A synthetase' (Dixon & Webb, 1958) and 'acetic thiokinase' [see *Biochem. J.* (1956), **64**, 782], catalyses the formation of acetyl-CoA from acetate and CoA in the presence of ATP. The enzyme from yeast has been studied by Jones, Black, Flynn & Lipmann (1953) and by Lynen (1953), and that from heart muscle by Beinert, Green, Hele, Hift, Korff & Ramakrishnan (1953), Korff (1953) and Hele (1954). For purposes of enzyme assay, the reaction has usually been followed by measurement of acetohydroxamic acid formation in the presence of hydroxylamine, of citrate formation in the presence of oxaloacetate and condensing enzyme, or by reduction of coenzyme I in the presence of malate, malate dehydrogenase and condensing enzyme. Beinert *et al.* (1953), however, describe briefly an assay of the enzyme in which the formation of acetyl-CoA is followed by the increase in extinction at 232 mμ (Stadtman, 1953). It seemed that such a method would be very suitable for the study of the enzyme in embryonic tissue. A procedure was tried out with extracts of acetone-dried mitochondria from livers of 12- to 14-day chicks as source of enzyme (see Experimental section), the system shown in Table 5 being chosen after consideration of the findings of the various workers cited above. The rate of increase of extinction at 232 mμ (ΔE/min.) was measured at room temperature in a Beckman model DU spectrophotometer. As ATP absorbs strongly at this wavelength, it was used in low concentration, and the reference cell contained all components of the reaction mixture except acetate. The reaction was started by addition of acetate solution to the experimental cell, an equal volume of water being added to the reference cell. Table 5 shows that CoA and ATP, as well as acetate, were essential for the activity, and that potassium chloride, magnesium sulphate and glutathione stimulated, in agreement with the various authors mentioned above. Sodium ions were inhibitory (cf. Korff, 1953): in a similar experiment to that in Table 5 the activity with the complete system was 7·01 units, and fell to 3·96 when the potassium acetate was replaced by an equivalent amount of the sodium salt. Fig. 2*B* shows that the system approached maximal velocity at a CoA concentration of 40 μM. With 22·5 μM, as used in the experiments so far described,

Table 5. *Cofactor requirements of* '*acetate-activating enzyme*' *in an extract of liver mitochondria from* 12- *to* 14-*day chicks*

Complete system: 0·02 M-tris chloride buffer, pH 8·5; 0·05 M-KCl; 66 μM-ATP; 0·33 mM-$MgSO_4$; 0·5 mM-glutathione; 3·3 mM-potassium acetate; 22·5 μM-coenzyme A; extract of mitochondria (34 μg. of N). Total vol., 3 ml. For other details see text.

System	Initial specific activity (ΔE_{232}/mg. of N/hr.)
Complete	4·06
Without KCl	1·88
Without ATP	0
Without $MgSO_4$	1·25
Without coenzyme A	0
Without glutathione	1·95
Without extract	0

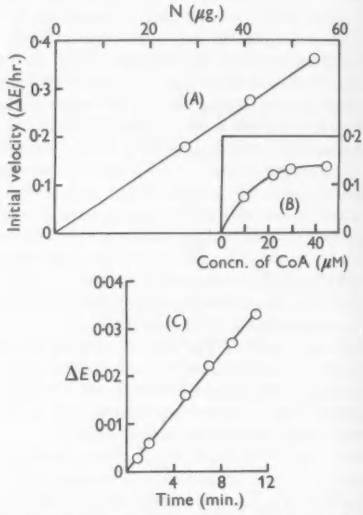

Fig. 2. 'Acetate-activating enzyme' in extract of mitochondria of 12-day chick liver. (*A*) Relationship between initial velocity and enzyme concentration. The abscissa represents the amount of extract (as μg. of nitrogen) present in the 3 ml. of reaction mixture; CoA, 45 μM; other constituents were as given in Table 5. (*B*) Relationship between initial velocity and CoA concentration. The system contained, in 3 ml., extract from 1·2 mg. of acetone-dried powder; other constituents were as given in Table 5. (*C*) Progress curve. The system included, in 3 ml., extract containing 26 μg. of nitrogen; CoA, 45 μM; other constituents were as given in Table 5.

progress curves were not linear; but when the CoA concentration was raised to 45 μM linear progress curves resulted (Fig. 2*C*) and activity was proportional to enzyme concentration (Fig. 2*A*). With this CoA concentration an assay method was thus achieved. It is not claimed, however, that the concentrations of the other components of the system are necessarily at their optimum values.

The relationship between activity and pH was ascertained with the tris chloride system as buffer from pH 6·9 to 9·2. Although frozen extracts appeared to be stable, some inactivation occurred during storage at 0°. The activity at one pH value (8·4) was therefore determined at intervals during the experiment and the activity at other pH values multiplied by appropriate factors to relate them to the activity of the freshly prepared extract. The total decrease in activity at pH 8·4 was not greater than 20%. The corrected curve (Fig. 3) shows two maxima, approximately at pH 7·8 and 8·4. Both were also obvious in the curve showing activity uncorrected for inactivation. Jones *et al.* (1953), using phosphate buffer and the hydroxamic acid method of assay, found a single optimum between pH 7·2 and 7·8.

Fig. 3 also shows an activity–pH curve obtained similarly to the other with an extract of mitochondria from livers of 16-day chick embryos. The same two optimum pH values are seen. The specific activity (ΔE/mg. of nitrogen/hr.) of the preparation from the embryos was considerably higher than that of the preparation from the hatched chicks (20·0 at pH 8·5 compared with 6·5). With another preparation from 16-day embryos the corresponding value was 20·8.

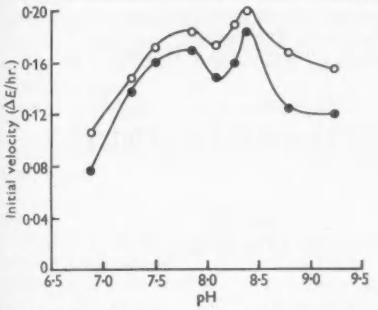

Fig. 3. 'Acetate-activating enzyme' in extracts from livers of 16-day chick embryos (○) and 12–14 day chicks (●); velocity–pH curves. The reaction mixture (3 ml.) contained embryo extract (9·6 μg. of nitrogen) from 0·16 mg. of powder, or chick extract (26 μg. of nitrogen) from 0·40 mg. of powder. CoA, 45 μM; other constituents were as given in Table 5. For other details see text. The activity at all pH values was constant for the first 15–20 min.

DISCUSSION

Great care has been taken throughout this work to avoid damaging the mitochondria. That their adenosine triphosphatase activity was stimulated by DNP may be taken as an indication that, at any rate, gross maltreatment has not been inflicted. The degree of such stimulation varies from one tissue to another; for example, Holton, Hülsmann, Myers & Slater (1957) found that, with addition of magnesium chloride, the ratio of the adenosine triphosphatase activities in the presence and the absence of 0·1 mM-DNP was sixteen for liver mitochondria and two for heart sarcosomes. With embryo mitochondria we found ratios of 2·2–3·5 (Table 3). It should be remarked that in experiments with mitochondria prepared in sucrose medium IV the incubation mixture contained no added magnesium chloride, whereas with the mitochondria isolated in the other three media it contained 0·5 mM-magnesium chloride, contributed by the mitochondrial suspension. Some isolation media, particularly those containing tris, yielded mitochondria with a higher adenosine triphosphatase activity in the absence of DNP than did others. The reason for this is not known. In contrast, Myers & Slater (1957) found that the presence of tris in the isolation medium decreased the adenosine triphosphatase activity of liver mitochondria. In our experiments, the high activity shown by particles isolated in some media would not seem to indicate that those media were particularly injurious, since the stimulation of the activity by DNP was of the same order in all cases.

Embryo mitochondria isolated in salt medium had an adenosine triphosphatase activity in the presence of 0·1 mM-DNP of about 50 μmoles of phosphate/mg. of nitrogen/hr. This may be compared with activities of 70–150 found by Lardy & Wellman (1953) for liver mitochondria maximally stimulated by DNP. Heart sarcosomes are twice as active as liver mitochondria after such stimulation (Holton *et al.* 1957).

Our observations with embryo mitochondria might be criticized on the ground that the particles were isolated from a mixture of tissues. This possible criticism is met to some extent by experiments with subdivided embryos reported in the next paper (Carey & Greville, 1959).

SUMMARY

1. Differential centrifuging of homogenates of whole 5-day chick embryos has been shown by distribution and other studies to give rise to cell fractions similar to those obtained with adult tissues, namely nuclei and unbroken tissue, mitochondria, microsomes and a soluble fraction.

2. The adenosine triphosphatase activity of the mitochondria, isolated in several different media, was stimulated by 2:4-dinitrophenol; this suggests that the mitochondria were not seriously damaged.

3. The absorption bands of the cytochrome components in the embryo mitochondria corresponded quite closely to those observed in liver mitochondria.

4. 'Acetate-activating enzyme' activity has been studied in extracts of the mitochondrial fraction from the livers of young chicks and of 16-day chick embryos.

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Mitochondria from Embryonic Tissues of the Chick

2. METABOLIC ACTIVITIES

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Needham (1931) produced evidence that during development the chick embryo uses its energy stores in a sequence: utilization of carbohydrate precedes that of protein, which in turn precedes that of fat. Results consistent with the view that carbohydrate is utilized in the early stages have been obtained in studies with chick embryos *in vitro*. Thus in the presence of glucose, embryos of 3–5 days' incubation show a high anaerobic glycolysis (Warburg, Posener & Negelein, 1924) and a respiratory quotient of unity (Dickens & Šimer, 1930, 1931; Needham, 1932*a*). Needham (1932*b*) concluded from measurements of am-

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monia production that about one-fifth of the respiration under these conditions is to be ascribed to the oxidation of protein. In the absence of added substrate the respiratory quotient falls to about 0·9, but the respiration is scarcely decreased during periods of up to 3 hr. (Dickens & Greville, 1933*a*). This intrinsic respiration can hardly be due to oxidation of carbohydrate, for the anaerobic glycolysis under these conditions is low (Dickens & Greville, 1932; Needham, Nowiński, Dixon & Cook, 1937), so that there seems to be little metabolizable carbohydrate in these embryos. Dickens & Greville (1933*a*) concluded that protein oxidation might account for rather less than one-half of the respiration in the absence of added substrate, and suggested that the embryos might contain reserve foodstuffs of unknown nature.

The large endogenous respiration may have been in part responsible for the failure of Greig, Munro & Elliott (1939) to show, by measurements of oxygen uptake, any oxidation of added tricarboxylic acid-cycle intermediates. It is also possible that these substances penetrated only very slowly into the cells of the embryos, which had been snipped with scissors. Succinate increased the respiration of homogenized embryos more than that of chopped ones (Elliott & Greig, 1938).

Spratt (1952) has shown a number of dehydrogenases, including those for pyruvate, 2-oxoglutarate, succinate and malate, to be present in chick blastoderms. It may also be mentioned that oxidation of intermediates of the tricarboxylic acid cycle has been observed with homogenates of eggs of the oyster *Ostrea commercialis* (Cleland, 1951) and of eggs of the sea urchin *Echinus esculentus* (Cleland & Rothschild, 1952). In early stages of cleavage of the eggs of the sea urchin *Arbacia*, however, the tricarboxylic acid cycle seems to be less important than the pentose phosphate pathway (Krahl, 1956).

The present work involved a study of the oxidation of intermediates of the tricarboxylic acid cycle and of substances concerned in fatty acid metabolism by mitochondria from 5-day chick embryos, the isolation and characterization of which are described in the preceding paper (Carey & Greville, 1959). Observations were extended to the mitochondrial fraction of livers of chicks in later stages of development.

EXPERIMENTAL

Materials

Water distilled in glass was used and, where possible, AnalaR reagents. Acidic substances were neutralized with NaOH before addition to media. As well as those described in the preceding paper (Cary & Greville, 1959), the following materials were used (m.p. values are uncorrected).

Sodium acetoacetate. Commercial methyl acetoacetate was purified through the bisulphite compound, and the fraction distilling at 79–80° (29 mm. Hg.) was hydrolysed in 0·1 M-soln. with NaOH according to the directions of Ljunggren (1924). The solution was concentrated to one-third of its volume in a rotary evaporator (bath temp., 25°; receiver in solid CO_2–ethylene glycol monoethyl ether) and then diluted to 0·1 M. A trial run with 0·1 M-methanol showed that 99·2% of this substance (determined by the method of Boos, 1948) was removed under these conditions.

cis-*Aconitic acid.* (i) *cis*-Aconitic anhydride, m.p. 76–77°, was prepared by the method of Malachowski, Giedroyć & Jerzmanowska (1928) and converted into the acid by the procedure of Krebs & Eggleston (1944). Despite repeated recrystallization from acetone–chloroform the m.p. could not be raised above 126–127°; Krebs & Eggleston (1944) give 130°. (ii) A sample of *cis*-aconitic anhydride (California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A.) had become converted into the acid during storage, to the extent of about 75%. It was extracted with chloroform at room temperature, and the residue recrystallized from acetone–chloroform; m.p. 126–127°. Aqueous solutions of both specimens of the acid remained colourless on addition of excess of alkali (cf. Krebs & Eggleston, 1944).

n-*Butyric acid.* This (supplied by British Drug Houses Ltd.) was distilled through a 25 cm. column. The material distilling at 161° was redistilled in the presence of $KMnO_4$, and the fraction of b.p. 161° (770 mm. Hg) collected.

Sodium crotonate. This was a purified sample prepared by Mr K. P. Harrison.

Sodium fumarate. Fumaric acid, recrystallized from ethanol–benzene, m.p. 286°, was converted into the sodium salt (loss at 125°, 0·2%).

Sodium D- *and* L-β-*hydroxybutyrate.* The specimens had been prepared by Lehninger & Greville (1953).

Sodium DL-*iso*citrate. DL-*iso*Citric acid lactone, m.p. 160–161° (California Foundation for Biochemical Research), was heated in 0·03 M-soln. for 15 min. on a boiling-water bath with 3·5 mol. prop. of NaOH. After being brought to pH 7·5 with HCl, the solution was concentrated *in vacuo* to 0·2 M.

Potassium DL-*lactate.* AnalaR lactic acid was diluted with water, and heated on a boiling-water bath for 30 min. and neutralized with KOH.

Disodium malonate. Commercial malonic acid was recrystallized from anhydrous ether–light petroleum (b.p. 60–80°). The acid, m.p. 135·5–136·5°, was neutralized with NaOH and the salt recrystallized from methanol–ethanol. (Found: loss at 130°, 10·8. Calc. for $C_3H_2O_4Na_2.H_2O$: H_2O, 10·8%.)

n-*Octanoic acid.* This (supplied by British Drug Houses Ltd.) was distilled through a 25 cm. column, and the fraction of b.p. 139·5–140° (27 mm. Hg) was collected.

2-*Oxoglutaric acid.* This was prepared by Mr B. R. Slater by the method of Friedman & Kosower (1946). A 0·2 M-soln. was stored at −10° and neutralized just before use.

Lithium pyruvate. This was prepared by the procedure of Wendel (1932) from freshly distilled pyruvic acid [b.p. 71–73° (19 mm. Hg)], and was 99·5% pure as determined by the carboxylase method.

Sodium pyruvate. This was prepared by Dr H. B. Stewart by a similar method, and was 99·2% pure as determined by carboxylase.

Coenzyme I ('cozymase 90', Sigma Chemical Co., St Louis, Mo., U.S.A.), coenzyme II (Sigma Chemical Co.), α-lipoic acid (Lederle Laboratories, Pearl River, New York, U.S.A.), L-malic acid (L. Light and Co. Ltd.) and L-glutamic acid (Roche Products Ltd.) were used without further purification.

Methods

The production of the embryos and the preparation of mitochondrial fractions from whole embryos and livers are described in the preceding paper (Carey & Greville, 1959).

Somite–nerve-cord preparations from 5-day embryos. Embryos were dissected under a lens; the head was removed and the viscera and the whole of the ventral body-wall, including the limb buds, were separated from the somite region. The tail bud was cut off, and the somites together with the nerve cord were stripped from the notocord. The tissue (0·1–0·2 g.) from eight embryos was homogenized in 0·5 ml. of salt medium (see below) in an ice-cooled homogenizer (Dounce, Witter, Monty, Pate & Cottone, 1955), which had a tube of 5 cm. length and 6 mm. internal diameter; 40–50 strokes were used. Nuclei and unbroken cells were removed by centrifuging for 5 min. at 600 *g*. The method of sedimenting the mitochondria, based on the principles of de Duve & Berthet (1953), was devised in collaboration with Dr J. B. Chappell, to whom we express our gratitude. The supernatant from the centrifuging at 600 *g* was centrifuged in tubes 2·5 cm. long which were placed in adapters in swing-out metal tubes in the MSE Major refrigerated centrifuge. The distances from the spindle to the surface and bottom of the fluid were 18·5 and 21·0 cm. respectively. Centrifuging for 10 min. at 3500 rev./min. (2600 *g* at 19·75 cm. from the spindle) was then enough to sediment the mitochondrial fraction, which was washed twice with 0·5 ml., and finally suspended in 0·65 ml. of the salt medium. Under the microscope the nuclear and mitochondrial fractions appeared similar to those from whole embryos (see preceding paper, Carey & Greville, 1959).

Preparation media. The media used in the cell fractionation were as follows.

Salt medium: 0·10 M-KCl; 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) chloride buffer, pH 7·5; mM-ethylenediaminetetra-acetic acid (EDTA); mM-adenosine triphosphate (ATP); 5 mM-$MgSO_4$ (Chappell & Perry, 1954).

Sucrose medium II: 0·29 M-sucrose; mM-EDTA; mM-ATP; 5 mM-$MgSO_4$.

Sucrose medium III: 0·31 M-sucrose, mM-EDTA.

Measurement of uptake of oxygen. This was determined at 25° in the Warburg apparatus, conical vessels of 7–8 ml. volume being used, with air as the gas phase; CO_2 was absorbed by 20% (w/v) KOH in the central tubes, the rims of which were treated with petroleum jelly. Diffusion of O_2 and absorption of CO_2 were shown to be adequate by control experiments with yeast. Mitochondrial suspensions were pipetted immediately after preparation into the manometer flasks, which already contained all other constituents; the first manometer readings were taken 1·5–2 hr. after opening the first egg.

Metabolic quotient. The symbol q_{O_2}, used throughout, denotes μl. of O_2 consumed/mg. of mitochondrial N/hr. Mitochondrial N was determined as described in part 1 (Carey & Greville, 1959).

RESULTS

Oxidation of intermediates of tricarboxylic acid cycle and related substances by mitochondrial fractions from 5-day embryos

Experiments were performed to test whether the tricarboxylic acid cycle is operative in mitochondria obtained from 5-day embryos. Table 1 shows that these particles could oxidize intermediates of the cycle, and also L-glutamate. The respiration was constant for 1 hr. or more except with fumarate, with which it increased during the first 30–40 min.; the q_{O_2} values for this substrate are for periods after the first 40 min. Citrate, *cis*-aconitate or DL-*iso*citrate supported only a very slow oxygen uptake.

Respiration in the presence of pyruvate (10 mM) was catalysed by addition of fumarate, L-malate or L-glutamate in low concentrations (mM or less). Some representative experiments are shown in Table 2. The rates of oxygen uptake were constant over the periods indicated. Sometimes pyruvate was oxidized rapidly in the absence of added catalyst, and this respiration could be so great that addition of catalyst caused no stimulation (Table 2, Expt. 5). It is possible that on these occasions endogenous substrates were present in amounts sufficient to catalyse the rapid oxidation of pyruvate; if so, they remained even after three washings of the particles.

As far as possible throughout this work, we have measured the respiration of each sample of mitochondria in the presence of 10 mM-pyruvate together with mM-fumarate as a check on the metabolic activity. The mean of the q_{O_2} values

Table 1. *Respiration of mitochondria from 5-day chick embryos*

The system (final vol., 0·5 ml.) contained mitochondria suspension (0·2 ml., 0·05–0·15 mg. of N), 10 mM-phosphate, pH 7·5 (Na^+, 16·8, K^+; 1·6 m-equiv./l.), mM-ATP (brought to pH 7·5 with NaOH), 5 mM-$MgSO_4$ and 10 mM-substrate, and was brought to 0·31 osmolar with sucrose (for mitochondria prepared in sucrose medium) or with KCl (for those made with salt medium). For other details see Experimental section. In a number of observations with no added substrate, the highest q_{O_2} value was 5.

Substrate	q_{O_2} Salt medium	q_{O_2} Sucrose medium II
2-Oxoglutarate	256, 164, 155	63
L-Glutamate	209, 191	75, 95, 77
Succinate	153, 295, 174	130, 184, 83
Fumarate	160, 114	—
L-Malate	117, 191, 154	32, 70, 57
Citrate	24, 30	—
cis-Aconitate	8, 0	—
DL-*iso*Citrate	15, 11	—

Table 2. *Mitochondria from 5-day chick embryos: catalysis by low concentrations of tricarboxylic acid-cycle intermediates of the oxidation of pyruvate*

For experimental details see Table 1.

Expt. no.	Preparation medium	Duration (min.)	Additions	q_{O_2}
1	Salt	100	(i) 10 mM-Pyruvate	48
		100	(ii) mM-Fumarate	27
		100	(i) + (ii)	198
2	Sucrose II	180	(i) 10 mM-Pyruvate	22
		180	(ii) 0·1 mM-Fumarate	0
		180	(i) + (ii)	70
		180	(i) + (ii) + 5 mM-malonate	33
3	Sucrose II	60	(i) 10 mM-Pyruvate	0
		60	(ii) 0·1 mM-L-Malate	0
		25	(i) + (ii)	79
		60	(i) + (ii) + 5 mM-malonate	0
4	Sucrose II	90	(i) 10 mM-Pyruvate	0
		90	(ii) mM-L-Glutamate	0
		90	(i) + (ii)	65
5	Salt	100	(i) 10 mM-Pyruvate	143
		100	(ii) mM-Fumarate	0
		100	(i) + (ii)	143

determined under these conditions with twenty-two preparations of mitochondria isolated in salt medium from 5-day embryos was $173 \pm 11{\cdot}4$ (S.E.).

The respiration in the presence of pyruvate and 0·1 mM-fumarate was inhibited by 5 mM-malonate (Table 2, Expts. 2 and 3). This is further evidence for the operation of the tricarboxylic acid cycle in these particles, since the concentration of malonate used was well below those stated by Peters (1952) to inhibit enzymes other than succinate dehydrogenase.

DL-Lactate (10 mM) was not oxidized when added alone or with the addition of fumarate (mM), coenzyme I (0·1 mM) and cytochrome *c* (0·02 mM) in various combinations.

The requirements for various cofactors for the oxidation of pyruvate in the presence of fumarate have been examined (Table 3). The mitochondria were washed once with 0·155 M-potassium chloride to remove components of the preparation medium. As considerable activity remained in the absence of added Mg^{2+} ions, the particles of another preparation were washed twice with 0·155 M-potassium chloride and then tested under conditions similar to those in Table 3. The activity was now completely dependent on the addition of Mg^{2+} ions: with no added magnesium sulphate the q_{O_2} was 0, with mM-magnesium sulphate, 73, with 2 mM, 98 and with 5 mM, again 98 (mitochondrial nitrogen, 0·08 mg./vessel). In a third experiment, the particles were isolated in sucrose medium II containing 5 mM-phosphate and washed twice with this solution. The q_{O_2} value for the whole system (Table 3) was 142 (mitochondrial nitrogen, 0·07 mg./vessel). On further addition of 0·1 mM-coenzyme I together with 0·1 mM-coenzyme II, 0·2 mM-α-lipoic acid, 0·1 mM-coenzyme A and mM-nicotinamide the q_{O_2} was 150. It was therefore concluded that addition of these various coenzymes, and also of cytochrome *c*, was without effect, whereas there was a definite requirement for Mg^{2+} ions and ATP.

Table 3. *Cofactor requirements for oxidation of pyruvate by mitochondria from 5-day chick embryos*

The tissue was homogenized in salt medium and the mitochondria were washed once with salt medium and once with 0·155 M-KCl. Whole system: 0·2 ml. of mitochondrial suspension (0·10 mg. of N); 10 mM-phosphate, pH 7·5; mM-ATP; 5 mM-$MgSO_4$; 20 μM-cytochrome *c*; 10 mM-pyruvate; mM-fumarate; KCl to bring to 0·31 osmolar. Final vol., 0·5 ml. Experimental period, 60 min.

	q_{O_2}
Whole system	151
Without cytochrome *c*	151
Without $MgSO_4$	99
Without ATP	10
Without substrates	0
Without mitochondria	0

The respiration of the mitochondria was completely inhibited, within the error of the experiments, by cyanide in low concentration (0·5 mM). The q_{O_2} values in the absence of inhibitor were 70 with malate as substrate, 95 with glutamate and 184 with succinate; in the presence of cyanide the q_{O_2} was in no case greater than 3. In these experiments the mitochondria were isolated in sucrose medium II and, to avoid loss of cyanide from the incubation medium, the potassium hydroxide in the central tubes was replaced by a calcium cyanide–calcium hydroxide suspension of the appropriate composition (Robbie, 1948).

The effects of 2:4-dinitrophenol on the respiration in the presence of pyruvate (10 mM) together with fumarate (mM) were very variable. With this agent in 0·01 mM-concentration there was acceleration with some preparations and inhibition with others. With lower concentrations, down to μM, accelerations up to 40% were observed. These findings suggest that the respiration of our particles is not severely and consistently limited as a result of the concomitant phosphorylation as it is with rat-liver mitochondria (Lardy & Wellman, 1952) and heart sarcosomes (Holton, Hülsmann, Myers & Slater, 1957), but that they behave more like the mitochondria from avian skeletal muscle (Chappell & Perry, 1953).

Oxidation of fatty acids and related substances by mitochondrial fractions from 5-day embryos

Needham (1931), as a result of his analysis of changes during development, indicated that fat oxidation is low in 5-day chick embryos (cf. Needham, 1942), and the respiratory quotient of unity observed *in vitro* (Dickens & Šimer, 1930; Needham, 1932*a*) is consistent with this. It need not, however, be assumed that the enzyme systems of fat oxidation are necessarily absent. Protein oxidation is also low at this stage, but observations on ammonia production have indicated that these embryos can oxidize their own protein *in vitro* if carbohydrate is lacking (Dickens & Greville, 1933*b*) or if carbohydrate breakdown is inhibited by fluoride (Needham, 1932*b*). It is therefore possible that enzymes of the fatty acid-oxidation system may be present even though they are not used *in vivo*. Since these enzymes are localized in the mitochondria, at any rate in adult mammals (Kennedy & Lehninger, 1948; Schneider, 1948), it seemed worth while to attempt in a few experiments to find out whether mitochondria from 5-day chick embryos would oxidize some of the substrates usually tested in studies of fatty acid oxidation. As judged from the oxygen uptakes, mitochondria prepared in salt medium failed completely to oxidize acetate (10 mM), butyrate (6 mM), octanoate (4 mM) or crotonate (10 mM) when those substances were added alone or in the presence of mM-fumarate. In these experiments the customary incubation medium was used (see Table 1), and respiration in the presence of pyruvate (10 mM) together with fumarate (mM) was of the usual order of magnitude.

Since the failure to obtain oxidation of fatty acids might be due to unsuitable technique, tests were made with other tissues. Mitochondrial fractions were isolated in salt medium from adult-rat and chicken livers, with the procedure and small quantity of tissue employed in the experiments with 5-day embryos. The rat-liver particles oxidized acetate, butyrate, crotonate and octanoate. For example, in one experiment (duration 60 min.; 0·23 mg. of mitochondrial nitrogen/vessel) the q_{O_2} values were as follows: with mM-fumarate, 70; with 0·8 mM-octanoate, 78; with 0·8 mM-octanoate plus mM-fumarate, 109; and with 4 mM-octanoate plus mM-fumarate, 165. Kennedy & Lehninger (1949) found that when rat liver was fractionated in isotonic saline the activity with respect to fatty acid oxidation was concentrated in the 'nuclear precipitate', whereas the 'large-granule fraction' was inactive, possibly as a result of osmotic damage. It now appears that if liver is fractionated in a potassium chloride medium containing the various other constituents introduced by Chappell & Perry (1954) (our 'salt medium') an active mitochondrial fraction is obtained.

The mitochondrial fraction from adult-chicken liver also oxidized butyrate, crotonate and octanoate, but only in the presence of catalyst. For example, in one experiment (duration 60 min.; 0·13 mg. of mitochondrial nitrogen/vessel) the q_{O_2} values were: with mM-octanoate or 10 mM-crotonate, 0; with mM-fumarate, 46; with mM-octanoate plus mM-fumarate, 86; and with 10 mM-crotonate plus mM-fumarate, 111. The results with adult-rat and chicken liver show that the technique was not grossly at fault.

The other substances related to fatty acids which were tested for oxidation by 5-day chick-embryo mitochondria were D- and L-β-hydroxybutyrate and acetoacetate (Table 4). [D(−)-β-Hydroxybutyric acid is the ketone body excreted in the urine in ketosis, whereas the L(+)-enantiomorph, in the form of its coenzyme A derivative, is an intermediate in the β-oxidation of butyric acid.] L-β-Hydroxybutyrate, either alone or in the presence of fumarate, was oxidized at a negligible rate by the embryo mitochondria, even when coenzyme A was added as well. On the other hand, the D-isomer was readily oxidized, and the rate was increased by fumarate in low concentration. It is likely that in the absence of fumarate this isomer suffered a one-stage dehydrogenation, catalysed by the D-β-hydroxybutyrate dehydrogenase and yielding acetoacetate. If this were so, the particles should have been able to oxidize added acetoacetate, but only at a low rate in the absence of fumarate. This was found to be the case (Table 4, Expts. 6 and 7). The method of preparation of acetoacetate probably left a trace of methanol in the product (see Methods). It was estimated that this would amount, on a molar basis, to not more than 0·8% of the acetoacetate, and hence to not more than 0·08 mM in the incubation medium. Addition of mM-methanol (to an extra vessel in Expt. 7, Table 4) did not increase the respiration in the presence of mM-fumarate.

Since the embryo mitochondria are capable of oxidizing ketone bodies, it might be that the fats of the yolk are converted into ketone bodies by the yolk sac, and that these are then transported to the embryo as a source of energy. This possibility was tested in two experiments, but no support was found for it. In the first, a homogenate of 5-day chick yolk sac in salt medium was used. The respiration in the presence of 10 mM-pyruvate plus mM-fumarate was 280 μl./hr./g. of tissue, but there was no oxygen uptake with mM-octanoate, either alone or in the presence of mM-fumarate. In the second experiment, the mitochondrial fraction was isolated from 5-day yolk sac in salt medium; the q_{O_2} value in the presence of 10 mM-pyruvate plus mM-fumarate was 143, but there was no oxygen uptake with mM-octanoate plus mM-fumarate or with 10 mM-crotonate plus mM-fumarate (duration 60 min.; 0·08 mg. of mitochondrial nitrogen/vessel).

Oxidation of fatty acids by mitochondrial fraction from livers of embryos and young chicks

Attempts to show oxidation of fatty acids by mitochondria from 5-day embryos having failed, attention was turned to the livers of older embryos. Embryos after 16–18 days' incubation were chosen, since they are said to oxidize fat almost exclusively (Needham, 1931, 1942). The results with the mitochondrial fraction isolated in salt medium are summarized in Table 5. With the exception of one observation with acetate, no oxidation of fatty acids was found under these conditions. As it had been shown to occur with similarly prepared particles from adult-chicken livers (see above), tests were extended to hatched young chicks. Oxidation of fatty acid was judged to occur when the initial rate of oxygen uptake with fatty acid plus fumarate was greater than that with fumarate alone, but often the two rates decreased almost to zero at about the same time. The rate of oxidation was never very great: the q_{O_2} value with fumarate plus fatty acid was not usually more than twice that with fumarate alone, and not more than half that with fumarate plus pyruvate. The findings (Table 5) suggest that, under the particular conditions, the older the chick the more likely it is that the liver mitochondrial fraction will oxidize fatty acids. The observations are few, but it seemed scarcely worth while to increase their number, because of the labour involved and also for reasons which emerged from work described below.

Table 4. *Mitochondria from 5-day chick embryos: q_{O_2} with acetoacetate and β-hydroxybutyrate enantiomorphs*

Mitochondria were prepared in salt medium. Mitochondrial N/vessel: 0·04–0·14 mg. For experimental conditions see Table 1; time, 50–110 min. Fumarate, mM; other substrates, 10 mM. In Expt. 5, addition of 0·16 mM-coenzyme A failed to evoke oxidation of L-β-hydroxybutyrate in the presence or absence of fumarate.

Expt. no.	Fumarate	Pyruvate + fumarate	L-β-Hydroxybutyrate	L-β-Hydroxybutyrate + fumarate	D-β-Hydroxybutyrate	D-β-Hydroxybutyrate + fumarate	Acetoacetate	Acetoacetate + fumarate
1	—	152	4	32	95	169	—	—
2	11	140	0	32	90	182	—	—
3	—	155	—	—	51	163	—	—
4	27	198	—	34	—	—	—	—
5	0	144	0	0	—	—	—	—
6	0	143	—	—	82	131	19	120
7	14	150	—	—	—	117	28	150

Table 5. *Oxidation of fatty acids by liver mitochondria of chicks at various stages of development*

Mitochondria were prepared in salt medium. 'Pos.' indicates that the substrate was oxidized, as judged from the oxygen uptake measurements, 'Neg.' that it was not, and the preceding figure gives the number of times such a result was obtained. A negative result was recorded if the difference between the q_{O_2} values in the presence and absence of fatty acid during the first hour was less than 5. In the 'Slight pos.' observation this difference was 17. Fumarate (mM) was present throughout. Acetate, 10 mM; butyrate, 4–6 mM; crotonate, 6–10 mM; octanoate, mM. The q_{O_2} values with 10 mM-pyruvate +mM-fumarate were between 170 and 370.

Stage of development	Acetate	Butyrate	Crotonate	Octanoate
Embryo, 16–18 days	7 Neg. 1 Pos.	1 Neg.	1 Neg.	4 Neg.
Chick, 1 day	—	3 Neg.	—	3 Neg.
Chick, 4–5½ days	—	1 Neg. 2 Pos.	1 Pos.	2 Neg. 1 Slight pos.
Chick, 12–14 days	2 Pos.	2 Pos.	1 Pos.	2 Neg.
Adult	—	1 Pos.	1 Pos.	3 Pos.

Oxidation of acetate by mitochondrial fractions from livers of 16- to 18-day embryos

Since the mitochondrial fraction of the livers of 16- to 18-day embryos could oxidize pyruvate, it could presumably oxidize acetyl-coenzyme A. It contained the 'acetate-activating enzyme' (Carey & Greville, 1959) and should form acetyl-coenzyme A from acetate. It might therefore be expected to oxidize acetate, and the usual failure to observe this (Table 5) needed investigation. It was found that the appearance of acetate oxidation was dependent on the nature of the medium in which the particles were prepared.

Of nine preparations in sucrose media, seven gave evidence of acetate oxidation and two did not. With these two, however, acetate oxidation became obvious after pre-incubation for 10 min. at 37° (Fig. 1). The untreated particles were kept at 0° while the pre-incubation took place, and the oxygen uptake was measured at 25° as usual. With the second of these preparations (Fig. 1*B*) the effect of pre-incubation was not so much to increase the respiration with acetate plus fumarate as to decrease the rate with fumarate alone, especially at first. This apparent evocation of acetate oxidation by pre-incubation, which we cannot explain, could not be studied further, because all the other preparations in sucrose media displayed acetate oxidation spontaneously, i.e. without pre-incubation. This treatment now introduced a lag in the respiration with both fumarate alone and fumarate plus acetate (Fig. 2) on four occasions: this may have resulted from breakdown of coenzymes which were resynthesized during the manometric observations. On two other occasions no lag appeared after pre-incubation but the curves with fumarate alone and fumarate plus acetate were brought closer together.

Of eight preparations in salt medium seven gave no evidence of acetate oxidation, and this could not be evoked by pre-incubation at 37°. It seemed possible that some constituent of the salt medium was preventing the demonstration of acetate oxidation. To test this, particles were isolated and washed twice in salt medium, and before the final centrifuging the suspension was divided into four equal portions. The particles were spun down and then severally resuspended in equal volumes of the following media: (*a*) 0·155 M-potassium chloride; (*b*) 0·11 M-potassium chloride plus mM-EDTA plus 0·05 M-tris chloride, pH 7·5; (*c*) 0·15 M-potassium chloride plus mM-ATP plus 5 mM-magnesium sulphate; (*d*) salt medium. The suspensions were left for 10 min. at 0°, and the uptakes of oxygen then determined with fumarate and fumarate plus acetate, at 25° as usual. The whole experiment was done three times (Table 6). The particles resus-

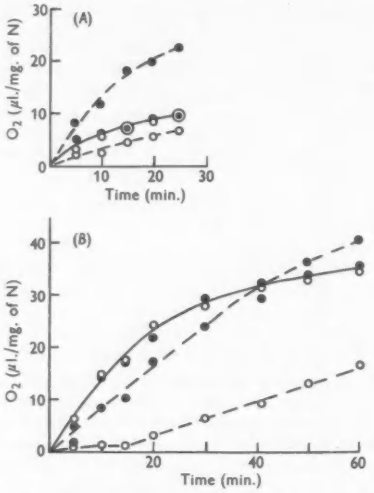

Fig. 1. Mitochondrial fraction from livers of 18-day chick embryos: effect of pre-incubation on respiration. Particles were isolated in sucrose medium III and washed three times. – – –, Particles pre-incubated for 10 min. at 37°; ——, untreated particles. ○, mM-Fumarate; ●, mM-fumarate + 10 mM-acetate. (*A*) Expt. 3/42, mg. of nitrogen/vessel: 0·43 (untreated particles), 0·32 (pre-incubated particles). (*B*) Expt. 3/43, mg. of nitrogen/vessel: 0·23.

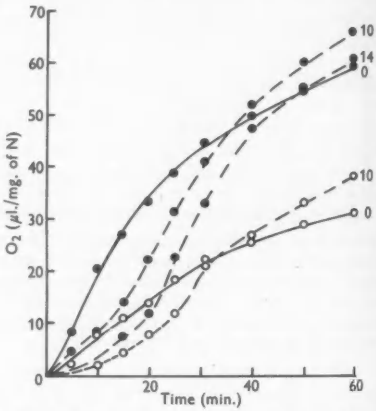

Fig. 2. Expt. 3/52. Particles were prepared as in experiments of Fig. 1. – – –, Particles pre-incubated at 37°; the numbers show duration of pre-incubation (min.). ——, Untreated particles. ○, mM-fumarate; ●, mM-fumarate + 10 mM-acetate. Milligrams of nitrogen/vessel: 0·35.

pended in medium (*b*), which contained EDTA and tris, behaved similarly to those resuspended in potassium chloride alone, whereas those resuspended in (*c*), which contained ATP and magnesium sulphate, behaved like those resuspended in salt medium. In the third experiment acetate increased the respiration with particles resuspended in salt medium; this was the sole occasion, recorded in Table 5, on which this happened; but the effect of acetate was much greater with particles resuspended in media (*a*) and (*b*).

It thus seems that it was the presence of ATP or magnesium sulphate, or both, in the salt medium which was mainly responsible for the relevant difference between this and potassium chloride solution. Circumstances prevented further analysis, so it is not known whether ATP or magnesium sulphate is effective alone. Table 6 shows that these substances reduced the apparent effect of acetate on the oxygen uptake as much by increasing the respiration in fumarate alone as by decreasing the respiration with fumarate plus acetate. It should be emphasized that it was arranged that the final concentrations of ATP (mM) and magnesium sulphate (5 mM) in the manometer vessels should be the same whichever medium was used for the resuspension. Conditions were not precisely the same in all vessels, however, since those used with particles resuspended in media (*b*) and (*d*) contained some EDTA (final concentration, 0·4 mM) and tris (20 mM) contributed by these media, for which no adjustment was made. Clearly, however, the presence or absence of EDTA and tris in the manometer vessels could not cause the differences under discussion, since these differences were observed between particles resuspended in medium (*a*) and those resuspended in medium (*c*), although the incubation mixtures were the same; and similarly for (*b*) and (*d*). It was the presence of ATP and magnesium sulphate in the medium in which the particles were resuspended after washing which caused the failure to show oxidation of acetate.

The presence of ATP and magnesium sulphate in the medium did not prevent the appearance of acetate oxidation with embryo-liver preparations made and finally suspended in sucrose medium, or with preparations from livers of adult birds made and finally suspended in salt medium.

It has been stated that of the preparations made in sucrose media seven showed acetate oxidation without pre-incubation and two did not. We could find no reason for this: after even more gentle homogenization and attempts to improve the temperature control during centrifuging, preparations still showed acetate oxidation without pre-incubation.

Mitochondrial fraction from subdivided 5-day embryos

Our work with 5-day embryos could be criticized on the ground that the mitochondria came from many tissues mixed together. The isolation and study of particles from individual tissues of such embryos would have required the development of special techniques. As a compromise some preparations were made from subdivided embryos. In the first few experiments 5-day embryos were crudely divided into two portions: (i) the head, snipped off just behind the hind-brain, together with the loosely hanging visceral organs, heart, blood vessels, etc., which were pulled away from the back; (ii) the nerve cord, notocord, somites, limb buds, body wall and closely adhering tissues such as the kidneys. The mitochondrial fractions

Table 6. *Mitochondrial fraction of liver of 16-day chick embryos: influence of suspending medium on respiration*

For experimental procedure see text. Fumarate, mM; acetate, 10 mM. Duration, 20 min. Mitochondrial suspension: 1 ml. was equivalent to 0·54 g. (expt. 1), 0·55 g. (expt. 2) and 0·63 g. (expt. 3) of liver.

Expt. no.	Final suspending medium	Uptake of O_2 (μl./ml. of suspension/hr.) Fumarate	Fumarate + acetate	Increase due to acetate (%)
1 (*a*)	KCl	50	86	72
(*b*)	KCl–EDTA–tris	69	112	62
(*c*)	KCl–ATP–$MgSO_4$	95	95	0
(*d*)	Salt medium	83	83	0
2 (*a*)	KCl	51	96	88
(*b*)	KCl–EDTA–tris	54	107	98
(*c*)	KCl–ATP–$MgSO_4$	80	80	0
(*d*)	Salt medium	69	69	0
3 (*a*)	KCl	36	138	283
(*b*)	KCl–EDTA–tris	50	140	180
(*c*)	KCl–ATP–$MgSO_4$	71	126	77
(*d*)	Salt medium	72	114	58

Table 7. *Respiration of somite–nerve-cord mitochondria from 5-day chick embryos*

For preparation of mitochondria see Experimental section. Experimental details are as given in Table 1; experimental period, 80 min. (i and ii); 70 min. (iii); 90 min. (iv). Fumarate, mM; other substrates, 10 mM.

	Uptake of O_2 (μl./ml. of mitochondrial suspension/hr.)			
	(i)	(ii)	(iii)	(iv)
Fumarate	3·2	—	4·7	—
Pyruvate + fumarate	16·0	20·8	27·0	23·0
D-β-Hydroxybutyrate	—	8·9	—	—
D-β-Hydroxybutyrate + fumarate	19·2	16·7	—	—
L-β-Hydroxybutyrate + fumarate	—	—	4·3	—
Acetoacetate	—	—	—	2·2
Acetoacetate + fumarate	—	—	—	15·3

prepared in salt medium from these two sources behaved similarly, in that the q_{O_2} value with 10 mM-pyruvate plus mM-fumarate was of the order of 200, and greater than the sum of the q_{O_2} values with 10 mM-pyruvate and mM-fumarate alone.

In further experiments particles were prepared from the somites together with the nerve cord as described in the Methods section. In the first of these the specific activity (μl. of O_2/ml. of mitochondrial suspension/hr.) was 6·5 with 10 mM-pyruvate, 21·3 with 10 mM-pyruvate plus 0·1 mM-fumarate and 8·9 with 10 mM-pyruvate plus 0·1 mM-fumarate plus 5 mM-malonate. Four further experiments (Table 7) showed that the behaviour of these particles towards the ketone bodies is similar to that of mitochondria from whole embryos (cf. Table 4). The findings with subdivided embryos indicate that the whole embryos give a general picture which could be used as a basis for further study with individual tissues.

DISCUSSION

Tricarboxylic acid cycle. The catalysis of the oxidation of pyruvate by tricarboxylic acid-cycle intermediates in low concentration was indicated by experiments (Table 2) in which the q_{O_2} value with pyruvate plus intermediate was greater than the sum of the q_{O_2} values with pyruvate and intermediate alone. This and the inhibition by 5 mM-malonate provide evidence that the cycle was operative in mitochondria from 5-day chick embryos. A peculiarity was the feeble oxidation of citrate and *cis*-aconitate by particles which readily oxidized other intermediates (Table 1). Possibly these two substances penetrate only very slowly into embryo mitochondria (cf. Plaut & Plaut, 1952 for heart-muscle particles). Rapid oxidation of DL-*iso*citrate was not to be expected since the unnatural isomer inhibits the metabolism of the *dextro*-enantiomorph (see Johnson & Lardy, 1958).

Oxidation of ketone bodies. Mitochondria from 5-day embryos oxidized acetoacetate in the presence of low concentrations of fumarate (Tables 4 and 7). Acetoacetate is oxidized readily by extrahepatic tissues of adult mammals but only very slowly by liver (Greville & Stewart, 1954; see also McCann, 1957). There is some evidence that it is oxidized by pigeon-breast-muscle mitochondria (McCann, 1957). We also found (Tables 4 and 7) that L-β-hydroxybutyrate was oxidized very slowly or not at all by mitochondria from 5-day embryos, whereas the D-enantiomorph was oxidized readily, the rate being increased by fumarate in low concentrations. Lehninger & Greville (1953) showed, with rat-liver particles, that the two isomers are oxidized through different pathways; McCann (1957) and Beechey (1957) have obtained further evidence for this with mitochondria from other tissues. The L-isomer is converted into its coenzyme A derivative and the latter oxidized to acetoacetyl-coenzyme A by L-β-hydroxybutyryl-coenzyme A dehydrogenase, which has been purified and characterized by Wakil, Green, Mii & Mahler (1954), Lynen & Wieland (1955) and Stern (1957*a*). The D-isomer is oxidized by D-β-hydroxybutyrate dehydrogenase (Green, Dewan & Leloir, 1937) to acetoacetate, which in extrahepatic tissues is converted into acetoacetyl-coenzyme A. The two pathways thus meet at the last-named compound. In liver, the D-isomer can also form a coenzyme A derivative (Lehninger & Greville, 1953), convertible into L-β-hydroxybutyryl-coenzyme A by a racemase (Stern, del Campillo & Lehninger, 1955) or by crotonase (Stern, 1957*b*). A D-β-hydroxybutyryl-coenzyme A dehydrogenase has also been claimed (Wakil, 1955). Thus a tissue which oxidizes acetoacetate might metabolize D-β-hydroxybutyrate by several routes: (i) dehydrogenation to acetoacetate and conversion of the latter into its coenzyme A derivative; (ii) conversion into D-β-hydroxybutyryl-coenzyme A followed by (*a*) racemization by racemase or crotonase and dehydrogenation to acetoacetyl-coenzyme A, or, possibly, (*b*) dehydrogenation to acetoacetyl-coenzyme A. Which of

these routes is followed in mitochondria from 5-day embryos cannot be decided without further experiments.

L-β-Hydroxybutyrate is oxidized by particles from liver, kidney (Lehninger & Greville, 1953; McCann, 1957) and heart (Beechey, 1957; McCann, 1957). In failing to oxidize this isomer, mitochondria from 5-day chick embryos would seem to resemble those from rat brain and pigeon-breast muscle (McCann, 1957).

Oxidation of fatty acids. Although we failed to observe any oxidation of fatty acids by mitochondria from 5-day embryos, in view of the finding that the oxidation of acetate by embryo-liver particles is dependent on the preparation medium and also on unidentified factors we would not claim that the first-named particles are incapable of oxidizing fatty acids. It is possible that the right conditions were not found.

In two experiments in which embryo-liver particles showed acetate oxidation after preparation in salt medium and resuspension in potassium chloride solution there was no indication of oxidation of octanoate. Again, a preparation which was made in sucrose medium and which showed acetate oxidation also failed to show oxidation of octanoate. Indeed, no oxidation of octanoate has been observed under any conditions, with one possible exception (Table 5), with particles from anything but adult livers.

In this work the ability of a preparation to oxidize a fatty acid was judged from the difference between (i) the rate of oxygen uptake with fatty acid plus fumarate and (ii) that with fumarate alone. With embryo-liver particles (ii) was rather high, and when acetate oxidation became obvious after some pretreatment of the particles this was sometimes due to increase of (i), sometimes to decrease of (ii) and sometimes to a combination of the two. Actual measurements of the oxidation of fatty acids would probably give more information with these particles than could be obtained by manometric observations. Determination of $^{14}CO_2$ produced from [^{14}C]fatty acids is likely to be the most practicable procedure. The present experiments, however, serve to illustrate the great effects which variations in preparative technique, sometimes even undetected ones, have on the subsequent respiration of the particles.

SUMMARY

1. A manometric study has been made of the oxidative metabolism of mitochondria from 5-day chick embryos.

2. The apparent oxidation of tricarboxylic acid-cycle intermediates and catalysis of the oxidation of pyruvate by fumarate and other intermediates, as well as the inhibition of the respiration by 5 mM-malonate, suggest that the tricarboxylic acid cycle was operative in these particles. DL-Lactate was not oxidized.

3. The respiration in the presence of fumarate plus pyruvate was dependent on addition of adenosine triphosphate and Mg^{2+} ions.

4. The respiration with various substrates was abolished by 0·5 mM-cyanide.

5. The mitochondria readily oxidized D-β-hydroxybutyrate and acetoacetate. Oxidation of L-β-hydroxybutyrate and fatty acids could not be demonstrated.

6. Experiments with preparations from subdivided embryos suggested that mitochondria from individual tissues would present a picture similar to that outlined above.

7. Manometric observations with mitochondrial fractions isolated in a salt medium from livers of embryos and chicks of various ages suggested that, under these conditions, the older the chick the more likely it is that fatty acid oxidation can be shown.

8. Oxidation of acetate by mitochondrial fractions from livers of 16–18-day chick embryos has been studied manometrically. Twice, particles isolated in a sucrose medium showed acetate oxidation only after pre-incubation in the absence of added substrate; subsequent preparations of this type showed it without pre-incubation. When particles isolated in a salt medium were suspended finally, before addition to the manometer vessels, in media containing magnesium sulphate and adenosine triphosphate, the apparent oxidation of acetate was much less than when they were finally suspended in media free from these substances. This was despite the presence of the two substances during the manometric observations in both cases.

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Distribution of Phosphorus Compounds in Blowfly Thoracic Muscle

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Watanabe & Williams (1951) showed that the sarcosomes in the flight muscle of the blowfly *Phormia regina* are in many respects homologous with the intracellular mitochondria of vertebrate tissue, and therefore it seemed likely that the sarcosomes were the site of oxidative phosphorylation in insect muscle. This process has been demonstrated *in vitro* in sarcosomes from the blowfly *Calliphora erythrocephala* (Lewis & Slater, 1954) and the housefly *Musca domestica* (Sacktor, 1954). The phosphorus:oxygen (P:O) ratios, however, were lower than those which have been reported for rat-heart sarcosomes (Slater & Holton, 1954) and for mouse-liver mitochondria (Kielley & Kielley, 1951). The reason for this difference is not clear. Siekivitz & Potter (1955), working with rat-liver mitochondria, showed that although low P:O ratios were associated with the loss of nucleotides, mitochondria which gave a P:O ratio as high as 2·0 when oxidizing succinate contained only about 5% of the total adenine nucleotides in the liver cells. Levenbook (1953), on the other hand, reported that 60% of the adenine nucleotides in the flight muscle of *P. regina* are retained by the isolated sarcosomes. In view of these findings we have attempted to identify the nucleotides and other phosphorus compounds in the thorax of *C. erythrocephala*, and have examined their distribution between the sarcosomes and the 'soluble' fraction from thoracic extracts. For this purpose we have used the 'labelled-pool' technique described by Winteringham, Bridges & Hellyer (1955), in which the phosphorus compounds are labelled *in vivo* with ^{32}P and subsequently isolated by paper chromatography.

MATERIALS AND METHODS

Adenosine triphosphate, adenosine diphosphate and muscle adenylic acid. These were purchased as the disodium salt, the sodium salt and the free acid respectively from Sigma Chemical Co., St Louis, Mo., U.S.A.

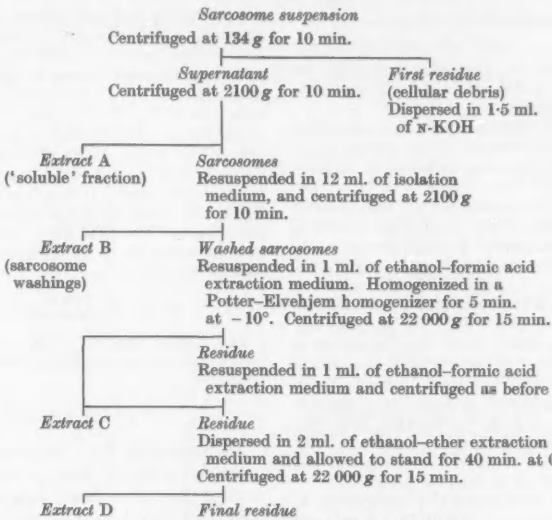
Sarcosome suspension
Centrifuged at 134 g for 10 min.
Supernatant
Centrifuged at 2100 g for 10 min.
First residue
(cellular debris)
Dispersed in 1·5 ml. of N-KOH
Extract A
('soluble' fraction)
Sarcosomes
Resuspended in 12 ml. of isolation medium, and centrifuged at 2100 g for 10 min.
Extract B
(sarcosome washings)
Washed sarcosomes
Resuspended in 1 ml. of ethanol–formic acid extraction medium. Homogenized in a Potter–Elvehjem homogenizer for 5 min. at −10°. Centrifuged at 22 000 g for 15 min.
Residue
Resuspended in 1 ml. of ethanol–formic acid extraction medium and centrifuged as before
Extract C
Residue
Dispersed in 2 ml. of ethanol–ether extraction medium and allowed to stand for 40 min. at 0°. Centrifuged at 22 000 g for 15 min.
Extract D
Final residue
Resuspended in 4·2 ml. of N-KOH.

Fig. 1. Fractionation of a sarcosome suspension of blowfly thoraces. The extracts were stored at −15° immediately after preparation.

Diphosphopyridine nucleotide. This was purchased from Sigma Chemical Co. and L. Light and Co.

Glucose 6-phosphate and 3-phosphoglyceric acid. These were purchased as the barium salts from Sigma Chemical Co. Before use they were dissolved in 0·1 N-HCl and the barium was removed by precipitation with an equivalent amount of sodium sulphate.

Sodium glycerophosphate. This was purchased from British Drug Houses Ltd.

Radioactive phosphorus. Carrier-free $H_3{}^{32}PO_4$ was purchased from The Radiochemical Centre, Amersham, Bucks.

Sarcosome-isolation medium. 0·318 M-Sucrose; 0·009 M-phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 7·4); 0·01 M-ethylenediaminetetra-acetate, pH 7·4.

Sarcosome-extraction media. (1) N-Formic acid in aq. 50% (v/v) ethanol. (2) Ethanol–ether (3:1, v/v).

Incorporation of [^{32}P]phosphate into the phosphorus compounds of the adult blowfly

Blowflies were reared at 23° and 80% relative humidity. Those used in the experiments were supplied with sucrose and water only. Flies were collected as required, immobilized by cooling, and placed in a 3 l. jar in which there were two small Petri dishes. One of these contained glucose and the other contained cotton wool saturated with 12 ml. of water containing 2 mc of [^{32}P]phosphate. Muslin was secured over the open end of the jar which was then kept at a constant temperature of 25°. The cotton wool was kept moist with water added from a pipette inserted through a small hole in the muslin. After a 48 hr. 'labelling' period the flies were transferred to another jar and were supplied with glucose and water only, and kept at 25° for an 'equilibration' period of 48 hr. unless otherwise stated.

Isolation and extraction of sarcosomes

Forty flies were collected in a conical flask and immobilized by cooling. Any [^{32}P]phosphate adhering to the flies was removed by washing them first in 5% ethanol containing 1% of KH_2PO_4, and then in aq. 1% KH_2PO_4 soln. They were then placed in an ice-cold dish lined with filter paper, which absorbed most of the moisture remaining on them. The heads and abdomina were removed, the thoraces collected in an ice-cold mortar and gently crushed in 3 ml. of isolation medium. The resulting brei was squeezed through six layers of muslin saturated with isolation medium, the filtrate being collected in a tube surrounded by crushed ice. A further 9 ml. of isolation medium was added to the residue, which was again squeezed and filtered. The combined filtrates (about 12 ml.) consisted of a suspension of sarcosomes and some broken myofibrils in a water-soluble thoracic extract, from which the sarcosomes were separated by differential centrifuging at 0° (see Fig. 1).

The isolated sarcosomes were homogenized at −15° in the ethanol–formic acid extraction medium, and the homogenate was centrifuged at 0° for 15 min. at 22 000 *g*. After a further extraction with ethanol–formic acid the residue was resuspended in ethanol–ether and allowed to stand for about 40 min. at 0° before being centrifuged. The details of the isolation and extraction procedures are given in Fig. 1. In one experiment a portion of extract *A*, and of extract *C* (Fig. 1), was treated with perchloric acid (final concn. 2%) and centrifuged at 0° for 10 min. at 22 000 *g*.

Paper-chromatographic techniques

Strips of Whatman no. 1 paper were soaked in 0·57 N-HCl in 45% ethanol for 30 min. They were then soaked for a further 30 min. in aq. 5% NH_3 soln. and finally washed in six changes of glass-distilled water.

Portions of each extract (50 μl. or 100 μl.) were applied to the filter paper strip, and dried in a stream of cold air and chromatographed by the ascending technique in partially evacuated glass columns (Winteringham *et al.* 1955). The following solvent systems were used, and will be referred to in the text as solvents 1 to 9. Their composition is given in vol. %, unless otherwise stated. 1. 98% Formic acid–acetone–water (15:50:35). 2. Acetone–15% (w/v) trichloroacetic acid (65:35). 3. Aq. 2 N-NH_3 soln.–methanol (30:70) (Burrows, Grylls & Harrison, 1952). 4. Methanol–98% formic acid–water (80:15:5). 5. Methanol–aq. NH_3 soln. (sp.gr. 0·88)–water (60:10:30) (Bandurski & Axelrod, 1951). 6. Phenol, water-saturated and containing 0·5% of 8-hydroxyquinoline (Hummel & Lindberg, 1949). 7. *iso*Propyl ether–90% formic acid (60:40) (Hanes & Isherwood, 1949). 8. 0·1 M-KH_2PO_4–Na_2HPO_4 (pH 6·8)–$(NH_4)_2SO_4$–propan-1-ol (100 ml.:60 g.:2 ml.) (Bock, Ling, Morell & Lipton, 1956). 9. *n*-Butyric acid–water–NaOH (69 ml.:31 ml.:0·85 g.) (Wade & Morgan, 1955).

Chromatograms were developed at 5° for periods of 40–50 hr. in solvents 1, 2, 4 and 5, and at 25° for the same periods in solvents 3, 6, 7 and 8. In solvent 9 chromatograms were developed at 20° for 5 days, and were run on Whatman no. 3 paper which had been washed with N-formic acid and with water as described by Wade & Morgan (1954).

Location of known 'marker' compounds on chromatograms. One of the criteria used in establishing the identity of a ^{32}P-labelled compound was coincidence on the chromatogram with a known 'marker' compound, which was chromatographed together with the ^{32}P-labelled material. When nucleotides had been added as markers they were located by their absorption under ultraviolet light. Chromatograms which had been developed in solvent 6 were washed with xylol and light petroleum to remove phenol, which masks absorption by nucleotides (Hummel & Lindberg, 1949). Other added phosphorus compounds were located with a molybdate reagent as described by Burrows *et al.* (1952).

Radiometric scanning of paper chromatograms. The ^{32}P-labelled compounds were located on the chromatograms as described by Winteringham, Harrison & Bridges (1952). The chromatogram is passed automatically beneath a thin-end-window Geiger–Müller tube and the radioactivity recorded graphically. Each zone of radioactivity on the strip is represented by a peak on the graph, and the area enclosed by each peak is proportional to the amount of ^{32}P in the zone which it represents.

Ultraviolet-absorption spectra. Some fractions were extracted from several chromatograms with 2% perchloric acid and the ultraviolet absorption of the extract was measured against that of a similar extract from an equal number of blank chromatograms.

Estimation of total phosphorus in sarcosome suspensions

The total phosphorus content of the sarcosome suspensions from '^{32}P-labelled' flies could not be determined, since these flies had been washed in a phosphate solution. An estimate of the phosphorus content of these suspensions was obtained by measuring the total phosphorus content of similar suspensions prepared from unwashed flies. A sample (0·1 ml.) of each suspension was heated together with 1 ml. of conc. HNO_3 for 30 min. and cooled. Concentrated H_2SO_4 (1 ml.) was added and the mixture heated to remove the HNO_3. After dilution with about 20 ml. of water the sample was boiled to convert any pyrophosphate into orthophosphate. It was then cooled, and made up to 20 ml. with water and its phosphorus content determined according to Milton & Waters (1955). Parallel determinations were carried out on equal volumes of the sarcosome-isolation medium.

Protein estimation

The protein content of the washed sarcosomes was determined by the method of Gornall, Bardawill & David (1949).

RESULTS

Distribution of ^{32}P. Sarcosome suspensions were prepared from ^{32}P-labelled flies, with the sarcosome-isolation medium, and then fractionated as shown in Fig. 1. Equal volumes of each extract were pipetted on to filter-paper disks. These were placed, in turn, beneath a thin-end-window Geiger-Müller tube, and the radioactivity was measured over a 5 min. period. The results of two such experiments, in which the relative amount of ^{32}P in each extract was calculated, are shown in Table 1. It will be seen that more than 90% of the ^{32}P is in extract *A* (the 'soluble' fraction) while the combined sarcosome extracts (*C*, *D* and final residue) contain less than 5% of the total activity. It will also be seen that the distribution of ^{32}P between the extracts is similar in both the experiments.

Identification of ^{32}P*-labelled compounds.* The phosphorus compounds in extracts *A*, *B*, *C* and *D* were separated into a number of fractions by chromatographing 50 μl. portions of these extracts in solvent 1. The distribution of these fractions along the chromatogram is shown graphically in Fig. 2. Each fraction except A_1 and C_1 (see below) was eluted at 5° from the chromatogram on to another strip, as described by Winteringham *et al.* (1955), and chromatographed in a second solvent. By repetition of this procedure we were able to study the chromatographic behaviour of the fractions from extracts *A*, *C* and *D* in a series of solvent systems. The R_p values obtained, and the suggested composition of the fractions based on coincidence with 'marker' compounds, are shown in Fig. 3. Further evidence for the identity of the ^{32}P-labelled compounds in the extracts, together with some data on the distribution of ^{32}P among the compounds in each fraction, is given below.

Table 1. *Distribution of* ^{32}P *among extracts* A, B, C, D *and final residue, derived from a sarcosome suspension as shown in Fig.* 1

The first residue (see Fig. 1), which contained 1·2% of the ^{32}P in the sarcosome suspension, is not included in this table. The count was from 20 μl. samples in Expt. 1, and from 10 μl. samples in Expt. 2.

		Vol. of extract (ml.)	Count/min.	^{32}P in each extract (as % of ^{32}P in supernatant from sarcosome suspension)
Expt. 1.	Extract *A*	11·6	11 009	92·03
	Extract *B*	11·6	427	3·56
	Extract *C*	2·1	1 261	1·90
	Extract *D*	1·4	1 728	1·74
	Final residue	4·2	255	0·77
Expt. 2.	Extract *A*	8·5	4 715	92·44
	Extract *B*	9·0	157	3·26
	Extract *C*	1·8	464	1·92
	Extract *D*	1·0	847	1·95
	Final residue	1·6	123	0·43

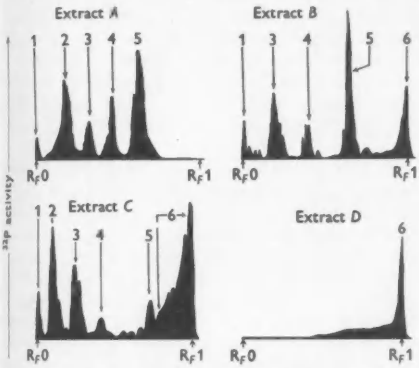

Fig. 2. Distribution of ^{32}P-labelled material on chromatograms after chromatographing extracts *A*, *B*, *C* and *D* in solvent 1.

Extract A

Fraction A_1. This fraction remained at R_F0 in solvent 1, and since it could not be eluted off the chromatogram further examination of its chromatographic properties in other solvents was not attempted. Even after the section of the chromatogram containing this fraction was heated in N-HCl at 100° for 7 min. no radioactivity could be detected in the hydrolysate. Furthermore the fraction was removed from the extract when this was deproteinized with perchloric acid.

We concluded from these results that fraction A_1 contained either phosphoprotein or compounds, such as nucleic acids, tightly bound to protein. When Winteringham *et al.* (1955) chromatographed extracts of '^{32}P-labelled' housefly thoraces in solvent 1, they also observed a fraction remaining at R_F0 which they attributed to phosphoprotein.

Fraction A_2. The adenosine triphosphate (ATP) fraction derived by chromatography of A_2 in solvent 6 was heated together with ATP in N-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1 it was found that the ^{32}P-labelled hydrolysis products coincided with those of ATP, and moreover that 66% of the ^{32}P coincided with orthophosphate. It was also found that the ultraviolet-absorption spectrum of A_2 was that of an adenine nucleotide. The chromatographic evidence indicated that over 90% of the ^{32}P in A_2 was accounted for by ATP, and about 6% by diphosphopyridine nucleotide (DPN).

Fraction A_3. After being chromatographed in solvent 3 this fraction was heated together with adenosine diphosphate (ADP) in N-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1 it was found that the ^{32}P-labelled hydrolysis products coincided with those of ADP and that 50% of the ^{32}P was released as orthophosphate. These findings provide support for the chromatographic evidence which suggested that all the ^{32}P in A_3 was accounted for by ADP.

Fraction A_4. When this fraction was chromatographed in solvent 3, it was resolved into three subfractions A_{4_1}, A_{4_2} and A_{4_3} (Fig. 3). A_{4_1} was again chromatographed in solvent 1, and on this occasion two portions (R_F 0·41 and 0·77) were obtained. The faster-running portion coincided with orthophosphate and accounted for 25% of the ^{32}P in A_4. The original chromatogram in solvent 1 showed that A_4 did not contain any orthophosphate. Therefore the orthophosphate in A_{4_1} must have been produced by the breakdown of a labile phosphorus compound in A_4. It is possible that this labile compound is arginine phosphate (Baldwin & Needham, 1934), since its behaviour in solvents 1 and 3 is the same as that of a compound identified as arginine phosphate by Winteringham *et al.* (1955). The slower-running fraction derived from A_{4_1} has not

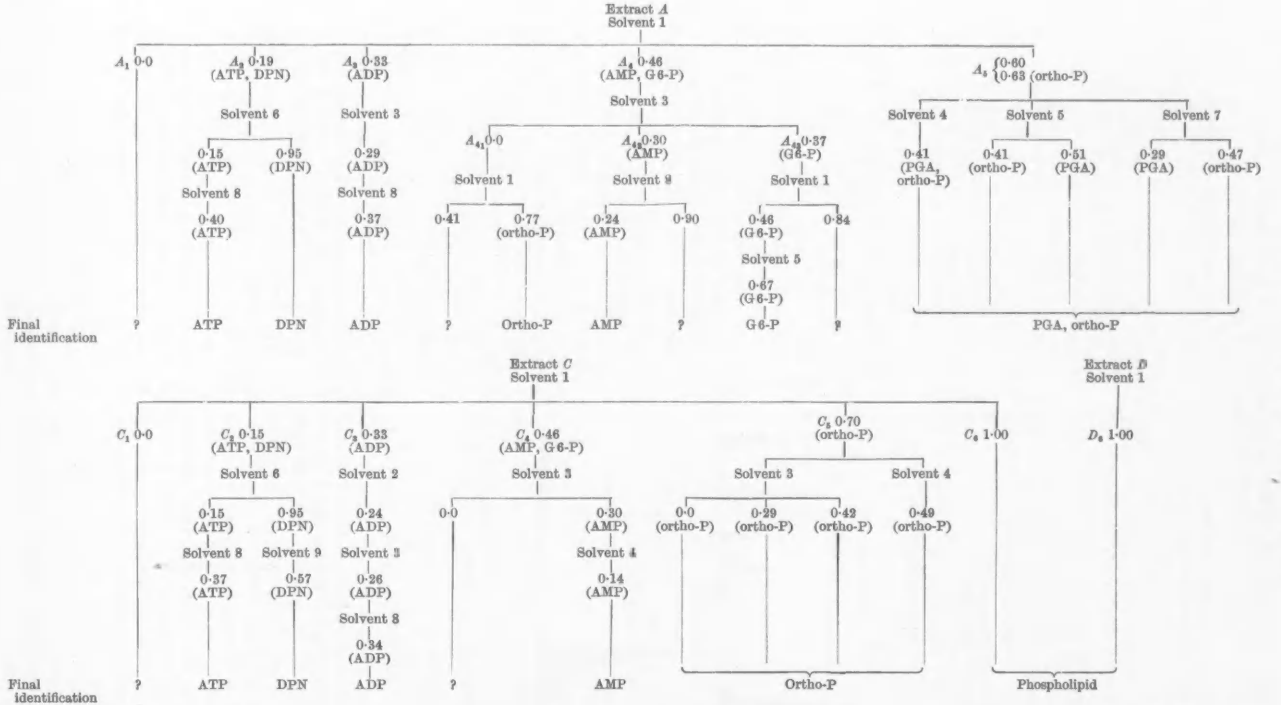

Fig. 3. Separation of ^{32}P-labelled compounds in extracts *A*, *C* and *D* by chromatography in a series of solvent systems. The solvent numbers and the R_F values of the labelled fractions are given, together with the marker compounds with which the labelled fractions coincided. The final identification is based on the chromatographic evidence shown in this figure and on the supplementary evidence given in the text. A_5 is composed of two subfractions which were never clearly separated in solvent 1. Ortho-P, orthophosphate.

been identified. It is not arginine phosphate since it was not hydrolysed by treatment with 0·01 N-HCl at 37° for 4 hr. (Mende & Chambers, 1953).

The chromatographic behaviour of the unidentified portion of A_{4_2} in solvent 1 (R_F 0·84) shows that it was not originally present in A_4 and therefore must have been produced by the breakdown of a labile compound in that fraction. We concluded that A_4 contains adenylic acid (AMP), glucose 6-phosphate (G 6-P) and at least two labile compounds, one of which is possibly arginine phosphate.

Fraction A_5. Orthophosphate accounted for 75% of the ^{32}P in this fraction, and the remainder was accounted for by 3-phosphoglyceric acid (PGA).

Extract C

Fraction C_1. This fraction resembles fraction A_1 in that it remained at R_F 0 in solvent 1, could not be eluted off the chromatogram, was insoluble in N-HCl and was precipitated when the extract was treated with perchloric acid. Therefore we concluded that C_1 also contains either phosphoprotein or compounds tightly bound to protein.

Fraction C_2. The 'ATP' fraction from solvent 6 (see Fig. 3) was heated together with ATP in N-HCl at 100° for 7 min. When the hydrolysate was chromatographed in solvent 1, the ^{32}P-labelled hydrolysis products coincided with those of ATP.

The 'DPN' fraction was heated together with DPN either in N-HCl at 100° for 7 min., or in N-NaOH at 25° for 18 hr. (Kaplan, Colowick & Barnes, 1951), and each hydrolysate was chromatographed in solvent 1. In both cases the DPN and the labelled fraction were completely hydrolysed, and the labelled hydrolysis products coincided with those of DPN.

The chromatographic evidence showed that 78% of the ^{32}P in C_2 is present in DPN and the remaining 22% in ATP.

Fraction C_3. After being chromatographed in solvent 8 this fraction was heated together with ADP in N-HCl at 100° for 7 min. and the hydrolysate was chromatographed in solvent 1. Two labelled fractions containing equal amounts of ^{32}P were obtained, and these coincided with the hydrolysis products of ADP. We concluded from the chromatographic evidence that the major component of fraction C_3 is ADP.

Fraction C_4. This fraction resembled A_4 in that it coincided with AMP and G 6-P in solvent 1. In solvents 3 and 4, however, 85% of the ^{32}P coincided with AMP, but none with G 6-P, which was separated from AMP in solvent 3. Therefore we concluded that the major component of fraction C_4 is AMP. The remaining 15% of this fraction has not been identified.

Fraction C_5. When this fraction was chromatographed in solvent 3, it was resolved into three fractions, each of which coincided with the KH_2PO_4 marker (Fig. 3). Three fractions were also obtained when ^{32}P-labelled orthophosphate was chromatographed together with KH_2PO_4 in this solvent. Curry (1953) has also observed the formation of several spots when orthophosphate is chromatographed in a neutral or an alkaline solvent, and has attributed this phenomenon to the separation of the three ionic forms. The fact that C_5 also gave rise to three fractions when chromatographed in solvent 3, and that each of these coincided with the phosphate marker, provides additional evidence that C_5 contains orthophosphate.

Fractions C_6 *and* D_6. C_6 is the largest fraction in extract *C*. However, the fact that in solvent 1, an acid solvent, it ran further than orthophosphate suggested that it contained compounds not usually found in acid-soluble extracts from muscle. This suggestion was substantiated by the fact that an even larger amount of a similar fraction accounted for most of the ^{32}P-labelled material in extract *D*, the ethanol–ether extract (Fig. 2). The rate of hydrolysis of fraction C_6 in N-HCl at 100° was followed by taking 50 µl. samples of the hydrolysate at intervals up to 6 hr. and chromatographing these in solvent 1. Only one labelled product was found, and this did not coincide either with orthophosphate or with fraction C_6. Furthermore, the amount of this product increased with time of hydrolysis (Fig. 4). Therefore it appeared that although C_6 was not removed from the chromatogram by boiling in N-HCl, it was slowly hydrolysed, with the production of a stable phosphorus compound which passed into solution. A similar compound was produced when fraction D_6 was hydrolysed in N-HCl. This finding, together with the behaviour of these fractions in solvent 1, suggested that their composition is similar and that they are

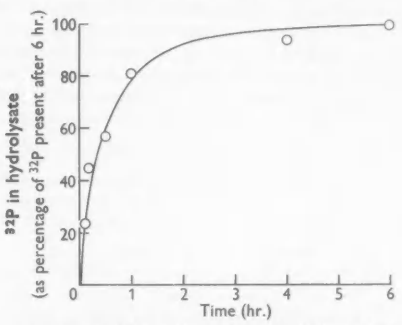

Fig. 4. Rate of liberation of ^{32}P-labelled hydrolysis product from fraction C_6 by N-HCl at 100°.

both derived from the same sarcosomal constituent, the greater part of which is extracted with the ethanol–ether medium. These observations suggested that fractions C_6 and D_6 contain phospholipids. This suggestion was supported by the fact that the ^{32}P-labelled hydrolysis product of both fractions coincided with glycerophosphate when chromatographed in solvents 1 and 3. It has been shown that the rate of hydrolysis of glycerophosphoric acid is markedly dependent on pH, and that it reaches a maximum at pH 4·0 (Bailly, 1942). The hydrolysates from both C_6 and D_6 were brought to pH 4·0, heated under 2 atm. pressure at 115° for 4 hr., and again chromatographed in solvents 1 and 3. After this treatment 74% of the labelled material coincided with orthophosphate in both solvents. This fact provided further evidence that the labelled hydrolysis product of both C_6 and D_6 is in fact glycerophosphoric acid. We concluded from the above evidence that both C_6 and D_6 contain phospholipids.

Extract B

This extract was obtained by washing the sarcosomes in the isolation medium. Although it has not been examined in detail, some indication of its composition was provided by chromatographing the extract in solvent 1 and comparing the radiochromatogram with those obtained from extracts *A*, *C* and *D*. The first fraction in extract *B* behaved like fractions A_1 and C_1 in that it remained at R_F 0. The second fraction coincided with ADP and the third with AMP, whereas in extracts *A* and *C* these compounds coincided with the third and fourth fractions respectively. This suggests that the second and third fractions in extract *B* correspond to fractions 3 and 4 respectively in extracts *A* and *C*, and that there is no fraction in extract *B* which corresponds to fraction 2, the fraction containing ATP and DPN in extracts *A* and *C*. The R_F value (0·65) of the last but one fraction in solvent 1 suggests that it corresponds to fraction 5 from extracts *A* and *C*, and therefore it may contain orthophosphate and PGA. The fact that the last fraction runs to the solvent front suggests that it corresponds to fraction 6 from extracts *C* and *D*.

Since extract *B* contains about 1·9 % of the ^{32}P in the supernatant from the sarcosome suspensions, its ^{32}P could not all be accounted for by contamination with extract *A*, because 0·5 ml. of this extract would be required to account for that amount of ^{32}P, and the volume of the sarcosome pellet was not more than 0·1 ml. Furthermore, the presence in extract *B* of fraction 6, a fraction not present in extract *A*, but present in *C* and *D*, indicates that extract *B* contains ^{32}P-labelled material derived from the sarcosomes.

Uniformity of labelling

In preliminary experiments with blowflies the distribution of ^{32}P was examined in extract *A* and in the ethanol–formic acid extract of unwashed sarcosomes, prepared from three groups of flies after a 'labelling' period of 24 or 48 hr., and an 'equilibration' period of 48 or 120 hr. The results given in Table 2 show that in extract *A*, and in the sarcosome extract, the distribution of ^{32}P among the major fractions is similar in all three groups. Only in extract *A* from flies labelled for 24 hr. is there an indication that equilibrium has not been reached. In view of this finding the flies used in all other experiments were supplied with ^{32}P for 48 hr. and allowed to metabolize for 48 hr. before being extracted.

It has already been shown that the ^{32}P-labelled ATP and ADP fractions from extract *A* are uniformly labelled, since on hydrolysis for 7 min. in N-HCl 66 and 50 % respectively of the total ^{32}P of these nucleotides was liberated as ^{32}P orthophosphate. Further evidence that equilibrium had been reached was obtained as follows. The total phosphorus content of the whole-muscle suspension prepared from 39 '^{32}P-labelled' flies was estimated to be 1·500 mg., which is the mean of four determinations (1·622, 1·295, 1·521, 1·565 mg.) made on sarcosome suspensions prepared from similar groups of 'unlabelled' flies (see Methods). With this value the amount of phosphorus in extract *A* was calculated on the basis of ^{32}P distribution. Further, by measurement of the areas enclosed by the peaks on the graphs we were able to calculate the percentage of phosphorus accounted for by the ATP

Table 2. *Effect of time on the distribution of* ^{32}P *in extract* A *and in an extract of unwashed sarcosomes*

Extract *A* and the unwashed sarcosomes were obtained as shown in Fig. 1. The fractions 1–5 (extract *A*) and 1-6 (ethanol–formic acid sarcosome extract) were obtained when these extracts were chromatographed in solvent 1 (Fig. 2). The ^{32}P in each fraction is expressed as a percentage of the ^{32}P in the extract.

'Labelling' period (hr.)	'Equilibration' period (hr.)	Extract *A*				Sarcosome extract				
		1	2+3	4	5	1	2+3	4	5	6
24	48	—	45·6	10·1	44·3	7·0	21·2	7·1	30·9	33·8
48	48	4·6	38·4	14·0	43·0	4·9	23·9	6·5	33·7	31·0
48	120	5·0	39·8	17·2	38·0	8·6	21·3	7·0	30·8	32·3

fraction in extract *A*, and from these data a value of 0·36 mM was obtained for the concentration of ATP in this extract. A second value for the concentration of ATP in extract *A* was obtained by a more conventional method. Preliminary experiments showed that by the procedure used to obtain the absorption spectra, 80 % of the ATP applied to a chromatogram could be recovered in the perchloric acid extract. After allowing for the 20 % loss during chromatographic and elution procedures, and by the use of a value of $14{\cdot}7 \times 10^3$ for the extinction coefficient (Pabst Laboratories, 1956), we obtained a value of 0·31 mM for the concentration of ATP in extract *A*. This value agrees closely with that calculated on the basis of ^{32}P distribution. Agreement between these two values could be obtained only if isotopic equilibrium had been reached and if the phosphorus compounds in the sarcosome suspension are uniformly labelled. Further evidence that this is so is the close agreement between the value of 4·8 μmoles/g. of sarcosomal protein for the DPN content of the sarcosomes, calculated on a ^{32}P-distribution basis, and the value of 4·7 μmoles/g. of protein which we obtained by the enzymic method of Racker (1950) as modified by Holton (1955).

The weights of the phosphorus compounds in the 'soluble' fraction (extract *A*) and in the washed sarcosomes (extracts *C* + *D*), calculated on the basis of ^{32}P distribution, are shown in Table 3.

DISCUSSION

It was shown by Winteringham *et al.* (1955) that the acid-soluble compounds in the thoracic muscle of the housefly, *Musca domestica*, can be uniformly labelled with ^{32}P when the insects are supplied with water containing carrier-free $^{32}PO_4^{3-}$. With blowflies we have found that the phosphorus compounds become uniformly labelled and that isotopic equilibrium is reached when the flies have been supplied with carrier-free $^{32}PO_4^{3-}$ for 48 hr. and kept for a further 48 hr. on a phosphate-free diet. Therefore we were able to use the distribution of ^{32}P as a basis for calculating the amounts of the major phosphorus compounds which have been identified in the muscle extracts.

Sarcosome suspensions were prepared from 'labelled' flies and the sarcosomes were isolated by differential centrifuging under conditions which would be expected to retain their ability to carry out oxidative phosphorylation (Lewis & Slater, 1954). The supernatant (extract *A*) represents the 'soluble' fraction and contains over 90 % of the phosphorus in the sarcosome suspension. Chromatographic analysis showed that the major phosphorus compounds in this extract were the same as those found by Winteringham *et al.* (1955) in housefly thoraces which had been extracted at −15°. Although arginine phosphate was not identified in our extracts, there was present in extract *A* a labile compound whose chromatographic behaviour resembled that of arginine phosphate. Compounds which have been identified in extract *A* include ATP, ADP, AMP, DPN, G6-P, PGA and orthophosphate. There are several other labelled compounds in this extract which were detectable on the chromatograms, but these have not been identified.

In the sarcosomes about 13 % of the phosphorus is accounted for by the nucleotides ADP, AMP and DPN. Much of the remaining phosphorus is accounted for by phospholipids, which are probably constituents of the sarcosome membrane, as has been suggested for rat-liver mitochondria by Siekivitz & Watson (1956). The composition of the sarcosome residue has not been examined, but it seems probable that it would contain nucleic acids, since only small amounts of these compounds would have been extracted with our media.

Levenbook (1953) has reported values, calculated on a dry-weight basis, for the adenine nucleotide content of the thoracic muscle of the blowfly *Phormia regina*. When these were recalculated on a wet-weight basis, a value of 72 % for the water content of the muscle being used (Levenbook & Williams, 1956), the following values were obtained (mg./g. of wet muscle): ATP, 4·55; ADP, 0·87; AMP, 1·13. We obtained comparable values (ATP, 4·1; ADP, 2·05; AMP, 0·51) by combining the amounts of adenine nucleotides in the soluble fraction and in the sarcosome extracts (Table 3). After measuring the absorption at 260 mμ of extracts of isolated sarcosomes, Levenbook (1953) concluded that 60–70 % of the adenine nucleotides in the muscle were present in the sarcosomes.

Table 3. *Distribution of phosphorus compounds in blowfly thoracic muscle*

The 'soluble' fraction (extract *A*) and the sarcosome extracts (*C* + *D*) were obtained as shown in Fig. 1. The phospholipid content of the sarcosomes was calculated by multiplying the value for 'phospholipid phosphorus' by 25.

Compound	Content (mg./g. of wet muscle) 'Soluble' fraction (extract *A*)	Sarcosome extracts (*C* + *D*)
ATP	4·09	0·01
ADP	2·01	0·04
AMP	0·45	0·06
DPN	0·50	0·06
G6-P	1·55	—
PGA	1·70	—
Phospholipid	—	1·91
PO_4^{3-}	3·10	0·02

This result is in contrast with our findings, which suggest that less than 3% of the adenine nucleotides are present in the sarcosomes, even if the nucleotides in extract *B* were derived solely from the sarcosomes.

Insect sarcosomes are believed to be homologous with vertebrate mitochondria. In order therefore to compare our values for the phosphorus compounds in blowfly sarcosomes with those of other workers for these compounds in vertebrate sarcosomes and mitochondria, we have recalculated our values (mg./g. of muscle in Table 3) in terms of μmoles/g. of sarcosomal protein. Our value for the DPN content (4·8 μmoles) is similar to that obtained by Holton (1955) for rat-heart sarcosomes (6·0 μmoles), and also to the values of Jacobson & Kaplan (1957) for rat-liver mitochondria and rat-kidney mitochondria (2·0 and 6·4 μmoles respectively). Our value of 4·2 μmoles for the ADP content is about one-fifth of that obtained by Slater & Holton (1953) for pig-heart sarcosomes (20·0 μmoles), but it should be noted that in the vertebrate preparation ADP was not estimated directly. In blowfly sarcosomes ATP and ADP together account for 6·1 μmoles of '7 min.-hydrolysable phosphorus'. This is about one-third of the amount (17·2 μmoles) found in mouse-liver mitochondria by Kielley & Kielley (1951). The value we obtained for inorganic phosphate (10·0 μmoles) is about one-sixth of that obtained by Cleland (1957) for rat-heart sarcosomes (64·0 μmoles). For AMP we obtained a value of 8·8 μmoles. The percentages of the total phosphorus of blowfly sarcosomes accounted for by acid-soluble phosphorus (24%) and by lipid phosphorus (60%) are similar to those accounted for (21 and 56% respectively) by these constituents in rat-liver mitochondria (Kennedy & Lehninger, 1949).

We have concluded that the phosphorus compounds in blowfly sarcosomes are similar to those found by other workers in vertebrate sarcosomes and mitochondria. However, the concentrations of the energy-rich compounds ADP and ATP in relation to protein are lower in the insect sarcosomes than in the vertebrate preparations.

SUMMARY

1. When blowflies (*Calliphora erythrocephala*) were supplied with $^{32}PO_4^{3-}$ for 48 hr. and left for a further 48 hr. on a phosphate-free diet, the phosphorus compounds in the fly became uniformly labelled and isotopic equilibrium was attained.

2. Sarcosome suspensions were prepared from the thoraces, and fractionated by differential centrifuging into a 'soluble' and a sarcosome fraction.

3. The sarcosomes were extracted with ethanol–formic acid and then with ethanol–ether.

4. The ^{32}P-labelled compounds in the 'soluble' fraction and in the sarcosome extracts were examined by paper-chromatographic techniques.

5. In the 'soluble' fraction, which contained over 90% of the phosphorus in the whole-muscle suspension, the following compounds were identified: adenosine tri- and di-phosphate, adenylic acid, diphosphopyridine nucleotide, glucose 6-phosphate, 3-phosphoglyceric acid and orthophosphate.

6. The sarcosomes contained adenosine tri- and di-phosphate, adenylic acid, diphosphopyridine nucleotide, orthophosphate and phospholipid.

7. A comparison is made of the amounts of phosphorus compounds in blowfly sarcosomes with those reported for vertebrate sarcosomes and mitochondria.

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Observations on the Molecular Weight and Chemical Composition of Nisin A

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Nisin is an antibiotic normally made up of a mixture of peptides containing the following amino acids: alanine, glycine, serine, aspartic acid, valine, methionine, leucine, isoleucine, histidine, lysine, proline, lanthionine and β-methyl-lanthionine.

Some of the single peptides received a preliminary investigation by Berridge, Newton & Abraham (1952), who worked with commercial material prepared at great cost for therapeutic experiments. Cheeseman & Berridge (1957) described a more economical method of preparation of material which was intended for structural investigations. This report describes the separation of the major peptide by countercurrent distribution, a determination of its molecular weight and the derivation of some possible amino acid sequences.

The determination of amino acids by elution from paper chromatograms was used to derive a minimum molecular weight. This was in good agreement with the maximum molecular weight calculated from diffusion coefficients. Thus a reasonable estimate for actual molecular weight could be made.

The molecule as a whole proved too complex for a total determination of the amino acid sequence at this stage, but the structures of several small portions of the polypeptide chain isolated by partial hydrolysis in acid were determined, end-group analysis being used.

EXPERIMENTAL

Solvents for countercurrent distribution. Unless otherwise stated, all solvents employed were of A.R. grade (British Drug Houses Ltd.). The butan-1-ol was tested before use for the presence of peroxides (Newton & Abraham, 1950), and was considered free of peroxides if no red colour developed when the alcohol was added to a freshly prepared solution of pure KSCN and $FeSO_4,(NH_4)SO_4,6H_2O$. Any positive samples found were redistilled over zinc powder before use.

Berridge *et al.* (1952) had used a solvent system consisting of methanol, butan-1-ol and acetate buffer at pH 3·0, in which nisin had an overall partition coefficient close to unity. This system had two advantages for its use. First, the partition coefficient at the beginning was close to unity; and other things being equal the maximum resolving power is obtained from a limited number of transfers when the geometric mean of the partition coefficients of the components of a mixture approaches unity (Craig & Craig, 1956). Secondly, at the pH used, some of the groups in the material appeared to be partly ionized and differences in solubility due to differences in the dissociation constants of the ionizable groups may occur under such conditions. The system had, however, a serious disadvantage from the preparative aspect which was that the nisin was not very soluble. It was therefore necessary to produce a new solvent which did not have this disadvantage, but retained the two advantages mentioned above. A mixture consisting of butan-1-ol, water, acetic acid and aq. NH_3 soln., with the pH of the aqueous phase at 2·8 to 3·0, was found to give satisfactory results. It was made by mixing 5 l. of water, 3·75 l. of butan-1-ol and 1·24 l. of acetic acid. The pH of the aqueous phase was adjusted by addition of conc. aq. NH_3 soln. to the mixture, and the solvent was kept overnight to reach equilibrium.

Apparatus. This consisted of approximately 100 units of normal design (cf. Craig, Hausmann, Ahrens & Harfenist, 1951), except that the second or decantation chamber (*B*) was similar to the first (*A*), having the same volume and having a decantation tube also at the same height (Fig. 1). Thus it was also possible to have bottom phase in chamber *B*. A complete cycle of operations therefore involved the addition of top phase to *A*, equilibration, separation, decantation of top phase into *B*, equilibration, separation and decantation. For one-half of the equilibrations the bottom phase in *B* was alone. This system had the advantage that for a given capital outlay and space practically double the usual number of units could be obtained, but there was the disadvantage that more transfers were required to effect a given separation than would be

necessary with the double number of Craig tubes. The apparatus gave a theoretical result with phthalic acid prepared from A.R. potassium hydrogen phthalate.

Preparation of nisin A

The sample of nisin used for this work was a solution of the bulked acetone precipitates, described in scheme 2 in the paper by Cheeseman & Berridge (1957). The nisin was in solution in 0·05 N-HCl and had a total activity of 87×10^6 units with a specific activity of 28 units/μg.

A portion of this solution, sufficient for three chambers of the countercurrent-distribution apparatus, was adjusted to contain the same concentration of butan-1-ol, aq. NH_3 soln., acetic acid and water as the bottom phase of the solvent mixture described above. The first three chambers of the apparatus were loaded and transfers begun, but it was necessary after each of the first ten transfers to centrifuge the contents of the chambers containing two layers to break the emulsion which proved difficult to separate. Interfacial precipitates which occurred during these initial stages were also removed after each centrifuging. After ten transfers the material remaining in the bottom layers of the first three chambers was removed and replaced by fresh bottom layer. The cycle of operations was continued for 336 transfers by which time the major peak of material was in chamber 85, giving a partition coefficient of K 0·68. The equation of Williamson & Craig (1947) for determining the partition coefficient was modified to make allowance for the double-chamber unit:

$$\frac{Tr}{Tr+2} = \frac{(r+2)\,(r+1)}{(n-r)\,(n+r+2)} \cdot \frac{4(K+1)}{K^2};$$

for example, in a given case, $A = r$ and $B = r+1$ (Fig. 1).

The contents of chambers 70–100, 101–126 and 127–152 inclusive were bulked to form three solutions. The material was recovered from each by precipitation with a method previously described in the preparation of nisin (Cheeseman & Berridge, 1957). In this method the solution was added to 2 vol. of 0·01 N-HCl and the mixture was then saturated with NaCl. The precipitated nisin separated at the interface between the aqueous and organic layers. This method was used in preference to the method of concentration and subsequent precipitation with acetone, as used by Berridge *et al.* (1952), as experiments had shown that losses of activity may occur when the earlier method is used.

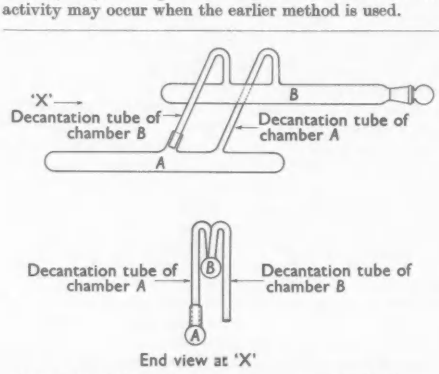


Fig. 1. Modified unit for countercurrent distribution.

The recovered nisin was redissolved in a few millilitres of 0·05 N-HCl and reprecipitated with 3 vol. of ice-cold acetone and centrifuged. The precipitate was resuspended in a few drops of water in order to remove the residual NaCl, nisin being of low solubility in saturated NaCl, and again centrifuged. The supernatant was discarded and the precipitate was dissolved in a minimum quantity of 0·05 N-HCl. This solution was heated to boiling for 1 min. for partial sterilization and stored in the refrigerator. When dry nisin was required it was precipitated with ice-cold acetone, washed with absolute acetone and dried *in vacuo*.

To confirm that the major peak isolated from the preparative experiments was a single component it was necessary to repeat the distribution in a different solvent system. A sample of the peak material was therefore subjected to distribution in the solvent mixture used by Berridge *et al.* (1952).

Two other similar experiments were carried out in order to produce sufficient material for further use.

Determination of amino acids

To afford the minimum destruction and loss of amino acids during hydrolysis it was necessary to use constant-boiling HCl that had been twice-distilled in glass apparatus to remove traces of metals which increase the rate of deamination of the amino acids (Jacobsen, 1949), and to carry out the hydrolysis under vacuum in a sealed tube.

A sample of nisin A weighing 59·8 mg. was hydrolysed in this way for 18 hr. at 105°, with 1 ml. of HCl. This was approximately the minimum time and temperature required for complete hydrolysis as indicated by the absence of peptides from paper chromatograms prepared from the mixture. These limits were chosen to ensure a minimum destruction of the amino acids. It is clear that other compounds with more resistant peptide bonds would need a longer hydrolysis. After drying *in vacuo* over P_2O_5 and KOH, the material was extracted five times with 5 ml. of ether to remove traces of ether-soluble material obtained by the breakdown of amino acids during hydrolysis, and the residue was dissolved in 10 ml. of 0·01 N-HCl. Measured portions (0·1 ml.) were spotted by means of an Agla micrometer syringe (Burroughs Wellcome and Co., London) on to Whatman no. 1 filter paper (18½ in. × 22½ in.). Paper chromatography was carried out according to the technique described by Levy & Chung (1953). After running and drying, the papers were heated for 30 min. at 85–90° to develop the fluorescence. The positions of the amino acids revealed under ultraviolet light were marked with pencil. One of the chromatograms from each tank was developed with ninhydrin to act as an additional control.

The marked areas of the amino acids were cut from the paper and each piece was cut into small strips and placed in a test tube. To each test tube was added 2 ml. of 1% $NaHCO_3$ soln., and, after 20 min. at room temperature, 1 ml. of ninhydrin reagent prepared by the method of Moore & Stein (1954*b*). The test tubes were then covered and heated in a boiling-water bath for 15 min. On cooling 5 ml. of ethanol–water (50:50, v/v) was added to each tube. The colour of the solutions was read on a Hilger Spekker instrument with the 606 yellow filter (601 violet for proline). A range of leucine standards was run for each experiment.

A mixture of amino acids of the kinds and quantities present in nisin was subjected to hydrolysis and chromato-

graphy exactly as was the nisin itself. The percentage loss of each amino acid was thus determined and the results obtained from the nisin hydrolysate were adjusted accordingly.

Determination of the diffusion coefficient

Diffusion coefficients (D) were determined in the Tiselius electrophoresis apparatus (Hilger), in which a cylindrical lens and an inclined knife edge are used to form the optical pattern representing the boundaries.

Nisin hydrochloride (peak A) in the form of a dried powder was dissolved in a solution containing HCl (0·05 M) and KCl (0·2 M), to eliminate diffusion potentials. The solution contained 232 mg. of nisin in 15 ml. and this formed the solution in the lower half of one side of the cell and in the whole of the other side.

Solvent was layered over the nisin on both sides so as partly to fill the sections above the U-tube. The two arms were levelled after the U-tube had been placed in the water bath (temperature 25°, closely controlled with a good toluene regulator and Sun-Vic proportioning head). The bottom section was slid into place, and the apparatus left for 3–4 hr. for temperature equilibration. The junction was then sharpened by the capillary method of Kahn & Polson (1947). Photographs of the boundary, taken at time intervals, were enlarged on to high-contrast paper (Ilford Ltd. Document no. 60) and traced on to transparent graph paper for measurement. The base line was also photographed, but its deviation from straightness was insignificant.

A test run with 0·25 N-KCl under these conditions gave 1·90, 1·91 and 1·94 $\times 10^{-5}$ cm.^{2}/sec. for D at different times. Gosting (1950), using more elaborate methods, reported values for D between 1·84 and 1·85 $\times 10^{-5}$ at this concentration and temperature. Thus the technique used for nisin would be accurate enough to discriminate between multiples of the minimum molecular weight which had been calculated from its amino acid content.

End-group analysis

N-group analysis with the 1-fluoro-2:4-dinitrobenzene (FDNB) method (Sanger, 1945, 1949) was carried out first on the purified intact nisin. Elliott (1952) suggested that groups which do not react with FDNB when the protein is insoluble may do so when it is soluble, and as nisin has a low solubility at the normal pH of the FDNB experiment, i.e. at pH 8–9, a further reaction was carried out at a pH at which nisin was readily soluble, i.e. about 2·5, the solution being treated with FDNB at room temperature for 72 hr. After hydrolysis of the dinitrophenyl (DNP)-nisin and the separation of the unsubstituted amino acids (Sanger, 1949), the DNP-amino acids were identified by paper chromatography (Blackburn & Lowther, 1951). A short period of hydrolysis (2 hr.) was carried out with one sample so that any acid-labile DNP-amino acids, e.g. DNP-glycine and DNP-proline, could be recognized (Sanger, 1945). *C*-group analysis was carried out with carboxypeptidase (Lens, 1949) from Armour Ltd. The enzyme was dissolved in alkaline solution and the pH was brought to 8·0 as soon as solution was complete (Harris, 1955). Nisin, being insoluble at the pH of the carboxypeptidase reaction, was therefore subjected to action of the enzyme in a solution containing 6 M-urea, the pH being adjusted to 8–9 by the addition of 1% trimethylamine. Under these conditions the nisin was completely soluble. It is probable that the molecule was also modified. Experiments on nisin suspensions in the absence of urea were also made.

Halsey & Neurath (1955) have shown that carboxypeptidase is active in 6 M-urea and that the urea may make the polypeptide or protein more readily available to the action of the enzyme by causing an unfolding of parts of the molecule. The enzyme experiments were carried out at 37° in the presence of di*iso*propyl phosphorofluoridate (DFP) (15 μl. of 0·1 M-DFP soln./mg. of enzyme) to inhibit any endopeptidase activity (Sanger & Thompson, 1953) and with an enzyme:substrate ratio of 1:50 (w/w).

Partial hydrolysis, fractionation and analysis

Nisin A (400 mg.) was hydrolysed in about 5 ml. of 6 N-HCl (glass-distilled constant-boiling mixture) for 5 days at 37°. The acid was then removed *in vacuo* over P_2O_5 and NaOH pellets. A second sample of 450 mg. was treated in the same manner, the products being worked up and analysed separately. The resulting amino acids and peptides were separated into fractions by elution chromatography on sulphonated-polystyrene cation-exchange resin (Dowex 50, cross-linked with 4% divinylbenzene and graded 200–400 mesh/in.). A satisfactory flow rate was obtained by removing coarse material with a 40-mesh/in. sieve and by removing fine material with a 270-mesh/in. sieve. The resin was prepared for use by the procedure of Moore & Stein (1951), and packed into a column (1·7 cm. diam., 2 m. long). The method of gradient elution described by Moore & Stein (1954*a*) was used with the same buffers except that it was necessary to add 2 N-NaOH gradually to the citrate–acetate buffer at the end of the run, to remove peptides which resisted elution at pH 5·1. Some of them even remained on the resin until the eluent was almost entirely 2 N-NaOH.

The positions of the various peaks in the eluate diagram (Fig. 4) were determined in the usual way, with the buffered ninhydrin reagent and 0·5 ml. samples from alternate tubes.

Each of the fractions so isolated was desalted and concentrated on a short column of Dowex 50X4 in the H^+ form, the amino acids and peptides being eluted with aq. 2 N-NH_3 soln. (Boulanger & Biserte, 1951; Partridge & Brimley, 1952).

The desalted fractions were subjected to two-dimensional paper chromatography (Whatman no. 3MM, 18½ in. × 22½ in.). The solvent used for the first dimension was butan-1-ol–water–acetic acid (4:5:1, by vol.) (Partridge, 1946), and that for the second, phenol–*m*-cresol (1:2, w/w) saturated with borate buffer, pH 8·3 (Levy & Chung, 1953). The paper was not, however, treated with buffer before the run in the second dimension as the presence of the buffer salts in the peptides to be eluted would have interfered with subsequent paper chromatography.

The positions of individual peptides and amino acids were marked after observation by fluorescence in ultraviolet light after heating the paper at 80–95° for 30 min., or, in some cases, by spraying with 0·025% ninhydrin, but this was usually avoided when the peptides were to be subjected to the DNP reaction.

A pointed section of paper containing each marked spot was cut out and the spot was washed towards the apex of the paper with 2% acetic acid. There it was concentrated by evaporation and finally eluted in a small volume. A

portion of the eluted material was dried, redissolved in a little 6N-HCl and hydrolysed in a sealed capillary overnight. The acid was then removed and the amino acids were identified mainly by two-dimensional paper chromatography as above. To reveal the amino acids a modification of the ninhydrin reagent similar to that of Levy & Chung (1953) was used. This consisted of ethanolic 0·2% ninhydrin–2:4:6-collidine–acetic acid (25:1:5, by vol.).

When sufficient peptide was available, amino end-group analyses were also made by the FDNB method of Sanger & Thompson (1953). The DNP-amino acids were identified either by paper chromatography in the *tert.*-amyl alcohol (2-methylbutan-2-ol) system (Blackburn & Lowther, 1951) or by reconversion into the free amino acid by heating with ammonia (Lowther, 1951) and subsequent paper chromatography.

Carboxypeptidase was used to reveal the *C*-terminal amino acids. Samples were taken at intervals and their subsequent investigation by paper chromatography enabled a determination of some *C*-terminal sequences to be made.

RESULTS

Homogeneity of the nisin

The distribution curves observed during purification (Fig. 2) suggested that only one major component was present. Relatively small changes in pH of the solvent mixture resulted in changes of the partition coefficient (K). When the pH was 2·8 K was 0·62, at pH 2·9 K was 0·68 and at pH 3·0 K was 0·72.

The value K 1·04 obtained in the analytical experiment (Fig. 3) is in close agreement with the partition coefficient K 1·05 which Berridge *et al.* (1952) obtained for nisin A. From the results in the two solvent mixtures it is probable that the material isolated is a single polypeptide and that it is identical with nisin A.

Amino acid composition of nisin

Table 1 summarizes the results obtained from the quantitative estimation of the amino acids in hydrolysed nisin. The recoveries of β-methyllanthionine and lanthionine have been based upon the average for the rest of the amino acids. The colour factors published by Moore & Stein (1954*b*) were used, except for the diamino acids, lanthionine and β-methyl-lanthionine, for which the factor 1·2 was chosen (cf. diaminopimelic acid). It was not necessary to confirm the factors of Moore & Stein (1954*b*) since discrepancies would be automatically 'ironed out' in adjusting the values according to the

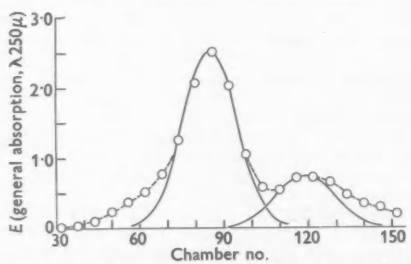


Fig. 2. Preparative countercurrent distribution of partially purified nisin. ○, Experimental results; continuous line, theoretical distribution.

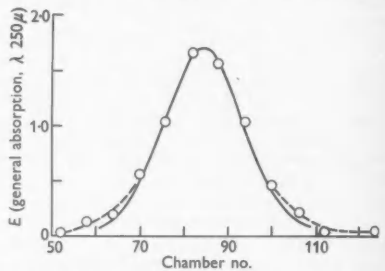


Fig. 3. Analytical countercurrent distribution of nisin A. ○, Experimental points; continuous line, theoretical distribution for K 1·04.

Table 1. *Amino acids and molecular weight of nisin A*

Amino acid	Concn. (g./100 g. of nisin)	Wt. of nisin to contain 1 mol.	No. of mol./mol. of nisin (assumed)	Mol. wt. of nisin from columns 3 and 4
Leu} Ileu}	16·85	778	9	—
Meth	6·41	2320	3	6960
Val	5·07	2310	3	6930
Pro	3·86	2980	2	5960
Ala	4·76	1870	4	7480
Gly	6·62	1130	6	6780
Ser	1·53	6870	1	6870
Asp	5·46	2440	3	7320
His	7·17	2170	3	6510
Lys	18·95	771	9	—
β-Lan	23·30	787	9	—
Lan	6·46	2550	3	—
	113·44		Mean	6826

yields of the whole process of hydrolysis and chromatography carried out on a known mixture simulating nisin.

Diffusion coefficient

Tracings from enlarged photographs of the schlieren diagram gave the results in Table 2, which shows the averages of values derived from some duplicate tracings and occasionally from different methods of mensuration.

On the third set of tracings calculations were made by the method of successive analysis at levels corresponding to steps of 0·146 cm. above the original boundary, i.e. on the dilute side. No significant trend in the value of D with dilution was discoverable.

It is clear that the values in Table 2 do not permit Longsworth's (1947) correction for zero time. This merely means that disturbances during the early history of the diffusion produced smaller errors than subsequent uncertainties. The figures may therefore be averaged, giving

$$D = 1{\cdot}63 \times 10^{-6} \text{ cm.}^2/\text{sec.}$$

Molecular weight

The average value for the minimum molecular weight, estimated from the quantities of most of the amino acids, is about 7000, the assumed number of residues being 55 (Table 1). As Tristram (1950) has indicated, in order to estimate the number of amino acid molecules in a protein or peptide the percentage error in the analysis of any one amino acid must not exceed $100 \times 0{\cdot}4/R$, where R is the number of residues of that amino acid. As this would require the errors for lysine, β-methyllanthionine and the leucines to be $\leqslant 4{\cdot}4\,\%$, these results were excluded when the average was taken. That for lanthionine was also excluded because of the uncertain recovery of this amino acid.

The maximum molecular weight of a protein-like molecule with a diffusion coefficient of $1{\cdot}63 \times 10^{-6}$ was calculated in the usual way, assuming the minimum reasonable value for f/f_0, namely 1·1 (Svedberg & Pedersen, 1940), and the likely value of 0·75 for the partial specific volume. The value obtained was 8500, which is near enough to 7000 to indicate that the molecule contains only one of the chemically determined units. A change of f/f_0 to 1·2 would more than compensate for the difference. We may therefore conclude that the molecular weight of nisin is approximately 7000, and that the molecule is approximately spherical in acid solution.

Table 2. *Diffusion coefficients of nisin A*

The values were obtained with a Tiselius electrophoresis apparatus (see text).

Diffusion time (sec.)	$10^{-6} \times D$ (cm.²/sec.)
15 840	1·56
26 730	1·60
43 590	1·69
86 220	1·65
117 060	1·67

End groups

No ether-soluble DNP-amino acids were obtained from DNP-nisin hydrolysed overnight, nor from a sample that had been hydrolysed for 2 hr. Increased time of treatment with FDNB and treatment at the lower pH still failed to produce any ether-soluble DNP-amino acids. The water-soluble DNP-amino acids were ε-DNP-lysine and imino-DNP-histidine. No free lysine would be detected in the hydrolysate. The reaction with histidine proceeded slowly but it was accelerated by prior treatment of the nisin with alkali at pH 8–9 for 2–4 hr. An increase in amino nitrogen (ninhydrin colour) also occurs whenever nisin is treated with alkali.

Although carboxypeptidase gave rapid and complete hydrolysis of the control peptide, glycylphenylalanine, no hydrolysis occurred with nisin. The enzyme–substrate mixture was subsequently kept for 72 hr., and the enzyme:substrate ratio was increased to 1:10 (w/w), but no free amino acids were observed on chromatograms of samples taken up to that time, and complete hydrolysis of the control peptide had occurred within 6 hr. The use of 6 M-urea as solvent, whilst affording increased solubility to the nisin, did not lead to the production of any *C*-terminal amino acids.

Composition of the partial hydrolysate

Table 3 indicates some of the peptides observed upon investigation of the 93 individual ninhydrin-positive regions found after two-dimensional chromatography of each of the 15 fractions obtained from the resin column (Fig. 4). Only those peptides which have been used for the derivation of amino acid sequences are noted in Table 3. A complete record is given by Cheeseman (1957). Two-dimensional chromatography was capable in most instances of separating the components of each fraction from the column into apparently pure amino acids and peptides. The sequence of the free amino acids from the column was the same as that described by Moore & Stein (1951). Aspartic acid, serine, glycine, alanine, valine, methionine, leucine, isoleucine and lysine were found as free amino acids in relatively large quantities. Proline, histidine, lanthionine and β-methyl-lanthionine were found free in small quantities only.

The material in fraction 14 was eluted from the column as the pH increased during the addition of

Table 3. *Analysis of peptides from partial hydrolysates*

Column, fraction no.	Paper subfraction no.	Amino acids after complete hydrolysis	Peptide structure deduced	Remarks
5	(2)	Asp + Gly + Val +	Gly.(Asp, Val)	By FDNB
5	(3)	Asp + + Gly + + Val + +	Gly.(Asp, Val)	By FDNB. Same R_F in phenol–cresol as 5 (2) but streaked in butanol–acetic acid. Probably same compound
5	(4)	Gly + + Ala + +	Gly.Ala	By FDNB
5	(6)	Asp + Gly + + Ala +	Ala.(Gly, Asp)	By FDNB
6	(2)	Meth + + Val + Gly + Ala + Lys +	(Gly, Ala, Lys)	Position of free Meth and Val, which therefore may or may not be included in the peptide
6	(4)	Lys + Gly ± Asp ± Ala ± Leu ±	Gly.(Lys, Asp, Ala, Leu)	By FDNB. The spot for DNP-Gly was weak. ε-DNP-Lys was positive
6	(7)	Gly + + + Ala + + +	Gly.Ala	By FDNB
6	(10) (11)	β-Lan + + + Gly + + +	β-Lan.Gly	Mono-DNP-β-Lan was observed
6	(12)	Lys + Asp + Ala +	(Lys, Asp, Ala)	Too little material for end-group analysis
6	(13)	Gly + + Ala + Meth + + Ileu + +	Ala.Gly.Ileu.Meth	By FDNB and carboxypeptidase
7	(3)	Asp + + Meth ±	Asp.Meth	By FDNB
7	(4)	Asp ± β-Lan + Gly ±	β-Lan.(Asp, Gly)	By FDNB
8	(1)	Ala + + + Meth + + + Leus + + +	Meth.Ala.Leus	By FDNB and carboxypeptidase
8	(4)	Gly + + + + Val + Meth + + +	Meth.Gly Val.Gly	Probably two peptides because both DNP-Meth and DNP-Val were obtained
10	(3)	Gly + + + Meth + + + Leu + + + Ala +	Leu.(Gly, Meth).Ala (?)	By FDNB but yield of DNP-Leu was very low. Ala was disregarded because of its relatively small quantity
14	(2)	Gly + Ser ± Asp ± Lan ± His ± Val ±	(Gly, Ser, Lan, His, Val)	Too little material for end-group analysis
14	(4c)	His + + + Val + + + Ileu + + + Ser + + +	(Ser, Ileu).His.Val	By carboxypeptidase
Z3	(4)	Asp + + + Ala + + +	Asp.Ala	From the second hydrolysate
Z6	(5)	Asp + + Lys + + Gly + +	(Asp, Lys).Gly	From the second hydrolysate

2N-NaOH to the citrate–acetate buffer. This material consisted mainly of large peptides containing the basic amino acids and the lanthionines. Fraction 15, which comprised the last part of the eluate, yielded only free amino acids. This was possibly because of the hydrolysis of residual peptides by the alkali. In some cases, for example in fraction 11, regions giving a faint ninhydrin reaction could be seen on paper chromatograms but on elution and hydrolysis the quantities of amino acids obtained were insufficient to permit confident recognition.

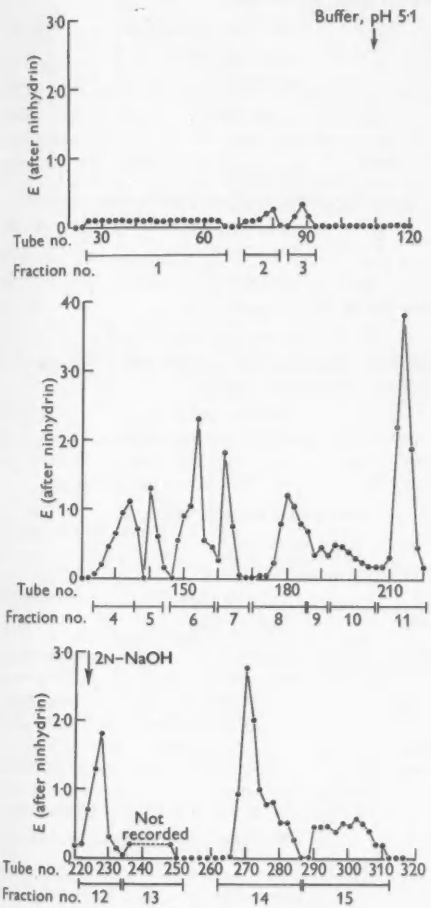


Fig. 4. Fractionation of partial hydrolysate on Dowex 50X4.

Derivation of sequences of amino acids

In deriving the sequences below it has been assumed that the β-carboxyl group in aspartic acid is not concerned in chain formation. No account has been taken of possible resynthesis of peptides during partial hydrolysis and subsequent separation. Except for one case in which the identity of a lanthionine spot is doubted, the results have been taken at their face value. We have arrived therefore at a few possible sequences. They are not contradicted by any of the numerous peptides which could be analysed for amino acids only, and not for sequence.

The sequences deduced represent only the first tentative step in unravelling a structure which could well be more complex even than that of bacitracin A, the molecule of which is less than a quarter of the size of nisin. Moreover, the bacitracin molecule has only three points of branching (Abraham, 1957), whereas in nisin the nine lanthionine and three β-methyl-lanthionine residues could give rise to 24 branches. The β-carboxyl groups of aspartic acid could increase this number by three.

The customary abbreviations (Brand & Edsall, 1947) for amino acids and peptides have been used. The abbreviations used for lanthionine and β-methyl-lanthionine are Lan and β-Lan. Leus is used to indicate that Leu or Ileu or both may be present.

From Table 3 we have the peptides

Asp_a.Meth	7 (3)
Asp_b.Ala	*Z* 3 (4)
(Asp_c, Lys).Gly	*Z* 6 (5)

As the aspartic acid residues can be distinguished by the amino acids which follow them, it is possible to label them as shown. There are only three of them in the nisin molecule. Therefore all sequences containing aspartic acid must agree with these three.

The peptide 5 (6) (Table 3) is either

Ala.Gly.Asp or Ala.Asp.Gly 5 (6)

whereas peptide *Z* 6 (5) is either

Asp_c.Lys.Gly or Lys.Asp_c.Gly *Z* 6 (5)

Thus 5 (6) may be part of Ala.Gly.Asp_c.Lys.Gly, this being the only way Asp_c could be included in peptide 5 (6). The same order is required for Asp_a and Asp_b. Therefore 5 (6) must be

Ala.Gly.Asp 5 (6)

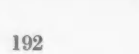
The peptide 5 (2) is Gly.(Asp, Val), but as no Asp may be followed by Val, it must be

Gly.Val.Asp 5 (2)

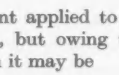
We also have

β-Lan.(Gly, Asp) 7 (4)

By the argument applied to 5 (6) this cannot be β-Lan.Asp.Gly, but owing to the two carboxyl groups of β-Lan it may be

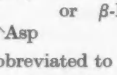
either  or

which will be abbreviated to

 7 (4)

Thus we also have the three aspartic acid residues accounted for in

Ala.Gly.Asp.[5 (6)], Gly.Val.Asp.[5 (2)]

and  [7 (4)]

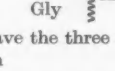
Asp_c must occur in one of these three peptides. Therefore lysine cannot precede aspartic acid and the only permissible arrangement for (Asp_c, Lys). Gly [*Z* 6 (5)] is

Asp_c.Lys.Gly *Z* 6 (5)

The peptide

Gly.(Lys, Asp, Ala, Leu) 6 (4)

contains an Asp residue, therefore one of the fragments Ala.Gly.Asp [5 (6)], Gly.Val.Asp [5 (2)]

or  [7 (4)]

must be present, at least partly, but Gly.Val.Asp 5 (2) cannot be present because Val cannot precede Asp in Gly.(Lys, Asp, Ala, Leu) [6 (4)]. If the Asp belongs to Ala.Gly.Asp [5 (6)], Ala must have been removed during hydrolysis and 6 (4) must have been part of the sequence

Ala.Gly.Asp.(Ala, Leu, Lys)

into which only Asp_b will fit, giving

Ala.Gly.Asp_b.Ala.(Leu, Lys) **A**

If now the other alternative that Asp in 6 (4) belongs to 7 (4) be considered, we arrive at

β-Lan.Gly.Asp_b.Ala.(Leu, Lys) **B**

by the same arguments as before. We have therefore these two possibilities for Asp_b.

It is possible to label the four alanine residues according to their positions in different fragments as follows:

Ala_a.Gly.Ileu.Meth	6 (13)
Ala_b.Gly.Asp	5 (6)
Meth.Ala_c.Leus [probably Ileu from 7 (1)]	8 (1)
Asp_b.Ala_d	*Z* 3 (4)

Ala_d being known to be different from the rest because it occurs in **A** or **B**.

Out of the six possible orders for the three amino acids in the peptide

(Lys, Asp, Ala) 6 (12)

the only one which agrees with sequences already deduced for aspartic acid is Asp.Ala.Lys, for Asp can be followed by Ala or Lys but cannot be preceded by [5 (6) 5 (2) or 7 (4)], and if Asp is followed by Lys the next residue must be Gly [*Z* 6 (5)], not Ala. This shows therefore that Asp in 6 (12) is Asp_b, and also gives the order of the bracketed components of **A** or **B**.

We have therefore

Ala.Gly.Asp_b.Ala_d.Lys.Leu

or β-Lan.Gly.Asp_b.Ala_d.Lys.Leu **C**

The six glycine molecules in nisin may be distinguished from one another by their adjacent amino acids, as follows:

Ala_a.Gly_a.Ileu.Meth	6 (13)
Ala_b.Gly_b.Asp	5 (6)
Meth.Gly_c	8 (4), 8 (6)
β-Lan.Gly_d	6 (10), 6 (11)
Val.Gly_e	8 (4), 8 (6)
Asp_c.Lys.Gly_f	*Z* 6 (5)

Similarly, with the three methionine residues:

Ala_a.Gly_a.Ileu.$Meth_a$	6 (13)
Asp_a.$Meth_b$	7 (3)
Leu.$Meth_c$.Gly_c	**D**

peptide **D** being derived from

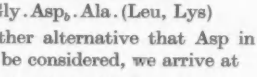
Leu.(Meth, Gly) 10 (3)

since Gly may have Meth preceding it but not Leu.

We have also the peptide

Meth.Ala_c.Leus, 8 (1)

which must therefore contain $Meth_a$ or $Meth_b$ and can extend Ala_a.Gly_a.Ileu.$Meth_a$ [6 (13)] or Asp_a.$Meth_b$ [7 (3)]. We may express this as

 **E**

The peptides

Gly.Ala 5 (4), 6 (7)

and (Gly, Ala, Lys) 6 (2)

cannot include Ala_c or Ala_d, as these are preceded by Meth and Asp_b respectively and if Ala comes first in 6 (2) the other amino acids would not agree with 8 (1) (Ala_c) or **A** or **B** (Ala_d). It follows that Ala_a or Ala_b, or both, are preceded by Gly. The Lys may then be directly before one Ala or before a Gly preceding Ala.

The single serine molecule occurs in the sequence

(Ser, Ileu).His.Val 14 (4*c*)

and in the peptide

(Gly, Ser, Asp, Lan, His, Val) 14 (2)

It can be shown that this peptide contradicts the sequences already deduced unless it be assumed

that in interpreting the chromatogram the faint spot designated lanthionine was in fact β-methyllanthionine. In this case the possible structures are numerous. The simplest which would agree with the previous deductions is Ileu.Ser.His.Val. β-Lan.Gly.Asp with a carboxyl and an amino group on β-Lan in unknown linkages. Leaving this as a mere possibility, we may summarize the sequences built up on definite evidence as follows, using sinuous lines to denote alternative sequences between which the choice cannot yet be made.

(1) (Ser, Ileu).His.Val 14 (4*c*)

(2) $Leu.Meth_c.Gly_e$ **D**

(3) β-Lan.Gly_d

Gly* ≀ $Asp_b.Ala_d.Lys.Leu$

(Lys, Gly) ≀ $Ala_b.Gly_b$ ≀

Lys **C**

When the choice indicated by the right-hand sinuous line in (3) can be decided the other alternative can be used in the sequence below, but at present both alternatives must be indicated in both (3) and (4). It is to be noted that the argument indicating the incorporation of β-Lan ≀ into Gly ≀ **C** implies the linkage β-Lan.Gly, but this is not so if those amino acids precede Asp_a or Asp_c as in (4) below.

(4) Gly*

(Lys, Gly) ≀ $Ala_b.Gly_b$ ≀ $Asp_c.Lys.Gly_f$ *Z* 6 (5)

Lys

β-Lan ≀

Gly_d ≀

Gly.Val ≀ $Asp_a.Meth_b$ ≀

Gly*

(Lys, Gly) ≀ $Ala_a.Gly_a.Ileu.Meth_a$ ≀ $Ala_c.Leus$

Lys **E**

These alternatives must remain open, because we know the three dipeptides which precede the three aspartic acid residues but, except that Gly.Val does not precede Asp_b [see argument concerning peptide 6 (4)], we do not know which dipeptide belongs to which aspartic acid.

The trace of alanine found in peptide 10 (3) was dismissed as too small to be significant. If it really was a constituent of the peptide the argument above is still valid and **D** would become

$$Leu.Meth_e.Gly_e.Ala_{a\ or\ b}$$

which would extend the sequence (4) in the left-hand direction.

* See 5 (4), 6 (7), 6 (2) just above and subsequent argument.

DISCUSSION

The partially purified nisin had a different composition from that obtained by Berridge *et al.* (1952). Of the two major peaks isolated by the previous authors only one was recovered as a major peak from the nisin distributed in these experiments. The possibility that the other part of the partially purified nisin, obtained as in scheme I of Cheeseman & Berridge (1957), could contain differing ratios of peaks, owing to differences in purification and extraction, has not been investigated.

Other biologically active material separated during the purification of the major peak confirms previous observations that the name nisin must be regarded as a collective one for a number of closely related polypeptides (Berridge, 1949, 1952; Hirsch, 1951; Bavin, Beach, Falconer & Friedmann, 1952; Berridge *et al.* 1952). In this respect nisin is similar to other polypeptide antibiotics which have also been found to occur as families, e.g. gramicidins, tyrocidines (Craig, Gregory & Barry, 1949), licheniformins (Callow & Work, 1952) and bacitracins (Newton & Abraham, 1950, 1953).

Within the limits of the methods employed the total quantities of amino acids recovered are in agreement with the estimated total weight. This would indicate that the molecule is comprised solely of amino acids or that if another compound is present it can only be so in relatively small amounts.

Although the recoveries by quantitative paper chromatography may be as low as 60% for some amino acids, the variations between the replicates of each estimation are less than 10%, permitting a confident approximate analysis. This is good enough when the number of residues per molecule of peptide is low. Similar techniques have been used before (e.g. Smith & Agiza, 1951). The small spread of the molecular weights deduced in Table 1 reflects the small variations in the analyses.

End-group analysis did not produce any evidence of *N*- or *C*-terminal amino acids. The ε-amino groups of all the lysine residues are free, but the slowness of the reaction of FDNB with the imino group of histidine suggests that this group is available only under alkaline conditions.

The lack of hydrolysis by carboxypeptidase does not necessarily indicate the absence of *C*-terminal groups. For example, the *C*-terminal amino acid may be present as proline, or an amide, e.g. glycine amide occurs in oxytocin (du Vigneaud, Ressler & Trippett, 1953), or it may have the D configuration (Neurath & Schwert, 1950) and so not be susceptible to enzyme action. The absence of both *N*- and *C*-terminal residues does, however, suggest the possibility of a closed polypeptide ring.

The amino acid sequences given above need confirmation since in some cases the results depend on single faint spots on chromatograms. Many peptides were present in quantities too small for end-group analysis. These have not been reported here, but only a little more information is needed to permit a choice between the alternatives indicated and to extend the sequences considerably. The branching of the polypeptide chain which may result from the presence of lanthionine and β-methyl-lanthionine could become very complex, as there are 12 of these molecules. This, the centre of interest in nisin, as it is possibly the centre of its antibiotic properties, has not been touched by the techniques of partial hydrolysis and end-group determination. An approach via the C–S–C linkages would probably be more fruitful.

SUMMARY

1. Nisin A has been purified and analysed for amino acids.

2. Its diffusion coefficient has been determined.

3. The agreement between the molecular weight of nisin calculated from its diffusion coefficient and that calculated from the proportion of various amino acids in the hydrolysate indicates that the molecule is approximately spherical in acid solution and that its weight is about 7000.

4. No amino or carboxyl end groups could be found, save the ε-group of lysine and the imino group of histidine.

5. From the analysis of partial hydrolysates a few possible amino acid sequences have been determined.

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Separation of Glycerylphosphoryl Inositol and Related Compounds on Ion-Exchange Columns

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No adequate procedures are at present available for a complete separation of the various types of glycerophosphatides. An accurate determination of some of the compounds present in such a crude phospholipid mixture can be achieved, however, by means of a mild alkaline hydrolysis, followed by two-dimensional paper chromatography of the resulting phosphoric acid diesters (Dawson, 1954). This method has been used in various studies of the turnover-rate of phosphate in different glycerophosphatides (e.g. Dawson, 1955; Hokin & Hokin, 1956; Ansell & Dohmen, 1956). The inositol-containing phosphoric acid esters obtained by Dawson's method were never identified. Furthermore, the constant detection of glycerophosphate highly labelled with ^{32}P, thought to arise from phosphatidic acid, is difficult to reconcile with the findings of some workers (Dawson, 1957; Marinetti, 1956; Marinetti & Stotz, 1956), who were unable to detect phosphatidic acid in animal tissues.

The present work extends Dawson's method by the use of ion-exchange resins instead of paper chromatography for the separation of the diesters. In this way it has been possible to study the conditions of hydrolysis more carefully and to investigate the structure of certain products of phospholipid hydrolysis, particularly that from monophosphoinositide, which does not run well on paper chromatograms.

A short note of this work has already appeared (Hawthorne & Hübscher, 1958).

MATERIALS AND METHODS

Preparation of phospholipids. Phosphatidylserine was prepared from ox-brain kephalin by the method of Folch (1942). The combined fractions III and IV were used without dialysing them. Lecithin was prepared by passage of crude brain or egg lecithin in 95% ethanol through an alumina column, according to Hanahan, Turner & Jayko (1951). Liver monophosphoinositide was prepared from a sample of pig-liver lipid kindly supplied by Viobin Laboratories, Monticello, Ill., U.S.A. The crude lipid was suspended in chloroform (250 g. in 1 l.), and filtered. Lipid was precipitated from the filtrate with 3 vol. of absolute ethanol. The precipitate was redissolved in 500 ml. of chloroform and precipitated with 1·3 l. of acetone. This step was repeated and the precipitate then taken up in 500 ml. of chloroform saturated with water. The solution was passed through a cellulose-powder column (Whatman Standard Grade; size 2 cm. × 25 cm.) to remove water-soluble impurities and the lipid was precipitated by the addition of 2 l. of absolute ethanol to the eluate. Two final precipitations were made from 300 ml. of chloroform with 470 ml. of absolute ethanol. Such a preparation usually contained monophosphoinositide and phosphatidylserine in equal amounts, and is referred to as crude liver inositide. In some cases an alternative way of washing out water-soluble impurities was used. This was based on the work of Folch, Lees & Sloane-Stanley (1954). The lipid was dissolved in 400 ml. of chloroform–methanol (2:1, v/v) and shaken with 80 ml. of 0·01 M-$CaCl_2$ solution. The water layer was separated off, methanol was added to the lower layer to make up the volume to 400 ml. and the extraction was repeated. Preparations of monophosphoinositide of a higher degree of purity were obtained by further fractionation on silicic acid columns (Hanahan, Dittmer & Warashina, 1957).

Other phosphoric acid esters. β-Glycerophosphoric acid was a commercial preparation. Inositol monophosphate was prepared from phytic acid by the method of McCormick & Carter (1952) and purified on a resin column as previously described (Hübscher & Hawthorne, 1957).

Ion-exchange resins. In the early work Nalcite SAR (−80+200 mesh) was used. This is a monofunctional strong-base polystyrene anion exchanger, with about 10% cross-linking (Dow Chemical Co., U.S.A.). Since Dowex 2 is an equivalent resin which is more readily available, the behaviour of this resin (8% cross-linked, −100+200 mesh) was also studied. Dowex 1 (2% cross-linked, −100+200 mesh) was used with most of the compounds investigated. This resin has the slight disadvantage of swelling as the salt concentration of the eluent is reduced. The columns were usually repacked therefore, after washing with 3 M-ammonium formate. The anion-exchange resins were used in the formate form and the columns were 1 cm. in diameter and 15 cm. high.

For the removal of cations from various solutions, Zeo-Karb 225 (−16+50 mesh) was used. It was converted into the hydrogen form before use by washing with 2 N-HCl and then water until the eluate was neutral. All distilled water used in this work was freed from ions by passage down a column of Bio-Deminrolit (The Permutit Co. Ltd.).

Removal of borate and formate from fractions containing organic phosphates. Since high concentrations of these ions interfere with the paper-chromatographic separation of the free inositol, glycerol and serine produced by acid hydrolysis of the phosphates (Hübscher & Hawthorne, 1957), they were removed. The pooled fractions were diluted with an equal volume of 0·005 M-sodium tetraborate and applied to a column of anion-exchange resin similar to the one from

which they were eluted. Under these conditions the phosphoric acid esters were retained on the column, but most of the borate passed through. The remaining borate was removed with 150 ml. of 0·01 M-formic acid and the phosphates were then eluted with the lowest concentration of formate which would easily remove them. This was usually 0·05 M-ammonium formate for the diesters. Five 50 ml. fractions were collected. Those containing phosphate were pooled and applied to a column of Zeo-Karb 225 (2 cm. × 30 cm.) to remove NH_4^+ ions. The column was washed with water until neutral. The total eluate was then concentrated *in vacuo* (at less than 20°) to about 50 ml. The remaining liquid was removed by freeze-drying.

Hydrolysis of phospholipids. The method is essentially that of Dawson (1954). An amount of phospholipid containing about 12 mg. of phosphorus was dissolved in 8 ml. of chloroform; 63 ml. of methanol, 9 ml. of water and 20 ml. of methanolic N-NaOH were added and the mixture was immediately incubated at 37° with occasional shaking. After 30 min. (or shorter times, according to the experiment) the hydrolysate was cooled in ice and diluted with 160 ml. of water. It was stirred with Zeo-Karb 226 (H^+ form) until neutral and the resin was removed by filtering through a funnel plugged with a little cotton wool. The resin was washed with two 50 ml. portions of water and the combined filtrate and washings were extracted with 250 ml. portions of carbon tetrachloride, ether and light petroleum (40–60°) successively. After the first or second extraction it was often necessary to centrifuge for a short time to clear the emulsion formed. A final extraction with 1 vol. of *iso*butanol and separation of the aqueous phase yielded a yellowish solution. This was filtered and made up to 500 ml. with water and sufficient sodium tetraborate solution to give a final borate concentration of 5 mM. Total phosphate was determined on this solution, which was then loaded directly on to a resin column. Inorganic phosphate was never detected.

Determination of glycerol. The chromotropic acid method was used, as described by Burton (1957), with omission of the chromatographic separation. Control experiments showed that inositol did not interfere and that HCl hydrolysates could be used directly, without removal of the acid. Glycerol standards were included with each determination.

Determination of nitrogen. The micro-Kjeldahl method was used, digestion being with a selenium–copper sulphate catalyst (K_2SO_4, 2 g.; $CuSO_4,5H_2O$, 4 g.; Se, 1·5 g.), and about 50 mg. of catalyst mixture with 2 ml. of conc. H_2SO_4 being used.

Other methods. Hydrolysis of phosphates, chromatography of serine, ethanolamine, choline and inositol, and determination of phosphate, borate and inositol have been described in a previous paper (Hübscher & Hawthorne, 1957).

RESULTS

Hydrolysis product of phosphatidyl choline

A sample of highly purified brain lecithin containing 12 mg. of P was hydrolysed as described, for 15 min., during which time 71 % of the phospholipid P became water-soluble. When this solution was applied to a Nalcite SAR column in 5 mM-sodium borate, 86 % of the total phosphate came straight through and the remaining phosphate was eluted with the first 30 ml. of a solution containing 5 mM-sodium borate and 0·01 M-ammonium formate. Similar results were obtained with a Dowex-1 resin column. It can be concluded that glycerylphosphoryl choline is not adsorbed on anion-exchange resins, even in the presence of borate ions.

Separation of the hydrolysis products of crude monophosphoinositide

As it was hoped that the net charge of glyceryl-phosphoryl serine (GPS) might be sufficiently different from that of the hydrolysis product of monophosphoinositide for separation on a resin column, the formic acid–ammonium formate system was first used for elution. A 500 mg. portion of partially purified liver inositide containing roughly equal proportions of phosphatidyl serine and monophosphoinositide was hydrolysed for 15 min. and the water-soluble phosphoric acid esters were applied to a Nalcite SAR column. After washing with water the column was eluted with formic acid–ammonium formate solutions of increasing concentration (see Fig. 1). The main peak, eluted with 0·01 M-formic acid–0·01 M-ammonium formate, contained 86 % of the phosphate applied to the column. An analysis of the peak showed that it contained, in addition to glycerol and phosphoric acid, both serine and inositol. With the second solvent (0·01 M-formic acid–0·03 M-sodium formate) monoesters of phosphoric acid (mainly glycerophosphate) were eluted. The third solvent (0·1 M-formic acid–0·5 M-sodium formate) would have eluted any diphosphates, but the small amount of phosphate ester obtained was not identified. Rechromatography of the main peak, either lower concentrations of formic acid and ammonium formate or ammonium formate at alkaline pH

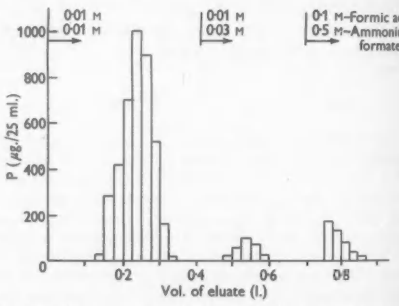

Fig. 1. Behaviour of crude liver inositide hydrolysate on a column of Nalcite SAR (−80 + 200 mesh, formate form), size 1 cm. × 20 cm. No borate was used in the elution. The large peak contained both GPI and GPS. Hydrolysis time, 15 min.

being used for elution, did not bring about a separation of the diesters contained in the main peak.

A separation of these diesters could be achieved by using the sodium tetraborate–ammonium formate system for elution (Khym & Cohn, 1954). In preliminary experiments it was found that the optimum concentration of borate for the separation of the various hydrolysis products was 5 mM. Such a separation is shown in Fig. 2. Peaks *B* and *C* were shown to be due to glycerylphosphoryl inositol (GPI) and GPS respectively. The isolation and analysis of these compounds are described below. Peak *D* contains glycerophosphate and there is some indication that peak *A* may be due to glycerylphosphoryl ethanolamine. In some experiments Nalcite SAR resin was used for the separation of the various phosphoric acid esters. Though essentially the same picture is obtained, slightly altered concentrations of ammonium formate were found necessary for elution. Thus GPI and GPS were eluted in the presence of borate with 0·06 M-ammonium formate and 0·08 M-ammonium formate respectively. Fig. 3 shows the isolation of GPI from purified monophosphoinositide and Fig. 4 the preparation of GPS from brain kephalin.

A time study of the hydrolysis was undertaken with the intention of explaining the presence of glycerophosphate (GP) in hydrolysates of tissue phospholipids. In Table 1 the relative amounts of the hydrolysis products are given. Three portions of partially purified liver inositide, each containing 10 mg. of phosphorus, were hydrolysed for periods of 15, 30 and 45 min. respectively. The amount of phosphate which became water-soluble during these periods was determined and the hydrolysis products were then separated on a Dowex-1 column. As can be seen from Table 1, the percentage of phosphate becoming water-soluble increased with time. The relative amounts of GP decreased slightly with time. This was due to the fact that the absolute amounts of GP isolated (2·44, 2·42 and 2·57 % of the original phospholipid P at 15, 30 and 45 min. respectively) remained constant throughout the hydrolysis. Further breakdown of GPI and GPS to GP is thus not likely to occur under the experimental conditions used. Consequently, the GP observed in the 15 min. hydrolysate cannot have been derived from these diesters.

Identification and characterization of glycerylphosphoryl inositol and glycerylphosphoryl serine

The fractions containing either of the diesters were pooled and treated as described above for the removal of borate and formate. The dry material was taken up in about 1 ml. of water. GPS was precipitated from this as a white solid after addition of 3 vol. of acetone. GPI was first converted into the barium salt by addition of freshly prepared aqueous barium hydroxide to neutrality and then precipitated by the addition of 10 vol. of acetone.

An analysis of GPI isolated from liver inositide on Nalcite SAR resin gave molar ratios for inositol:phosphoric acid:glycerol of 1·1:1:1. A sample of GPI isolated as the barium salt after

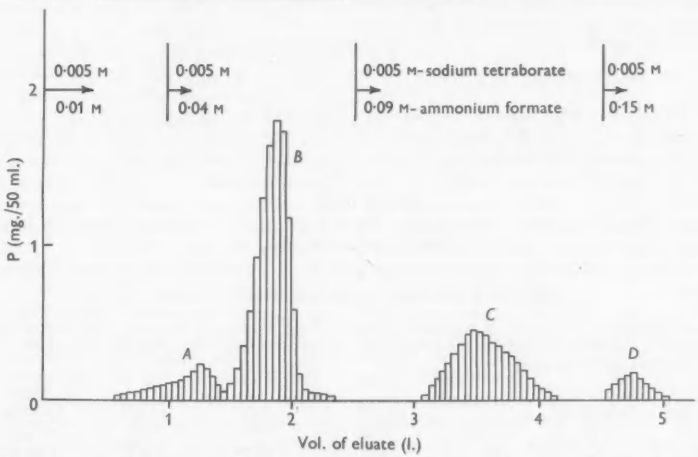

Fig. 2. Crude liver inositide hydrolysate fractionated on a column of Dowex 1 × 2 (100–200 mesh, formate form), size 1 cm. × 20 cm. Eluted with the sodium borate–ammonium formate system. Peak *A* probably represents glycerylphosphoryl ethanolamine, peak *B* represents GPI, peak *C* GPS and peak *D* glycerophosphate. Hydrolysis time, 30 min.

separation on a Dowex-1 column gave the following analysis after drying to constant weight at 50° *in vacuo* over P_2O_5 (Found: P, 8·1; glycerol, 29·1%; molar ratio P:glycerol, 1:1·19. The anhydrous barium salt of GPI requires P, 7·7; glycerol, 22·9, molar ratio 1:1).

Paper chromatography with the solvents described below showed about 12% of glycerophosphate to be present as an impurity. This probably arose from partial hydrolysis of GPI during isolation. Use of much milder isolation conditions (after borate removal, concentration *in vacuo*

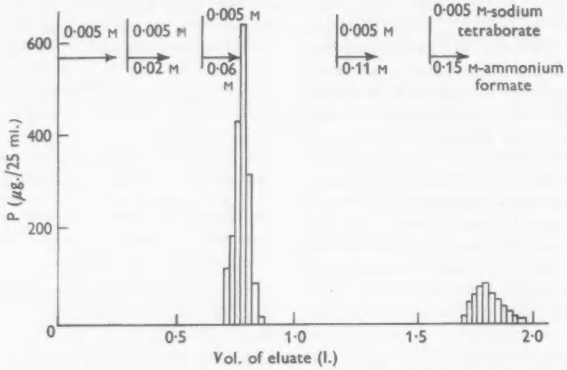

Fig. 3. Purified liver inositide hydrolysate fractionated on Nalcite SAR. Column details are as described in Fig. 1. The main peak is GPI. Hydrolysis time, 25 min.

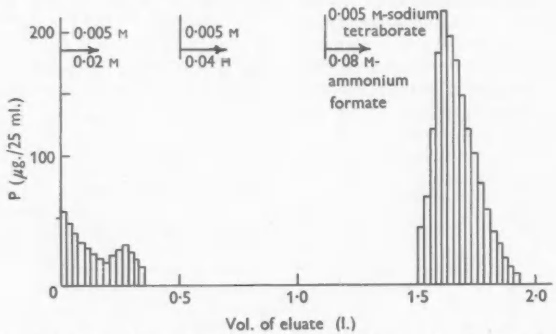

Fig. 4. Ox-brain phosphatidyl serine hydrolysate. Nalcite SAR column, as in Fig. 1. The main peak is GPS. Hydrolysis time 30 min.

Table 1. *Time study of phospholipid hydrolysis*

Samples of partially purified liver inositide containing roughly 10 mg. of phosphorus were hydrolysed as described under Methods. The figures represent percentages of the total water-soluble phosphates obtained after hydrolysis. 'GPE' has not been identified with certainty (see text).

Time of hydrolysis (min.)	Phospholipid hydrolysed (%)	'GPE' (%)	GPI (%)	GPS (%)	GP (%)	P not eluted (%)
15	42	8·5	45·7	24·2	5·8	15·8
30	57·5	6·1	49·0	26·3	4·2	14·4
45*	66	8·8	42·2	27·2	3·9	12·7

* An additional compound to those listed above was eluted with 5 mM-sodium borate and 0·09 M-ammonium formate, representing 5·2% of the total water-soluble phosphates.

only to 200 ml., then freeze-drying to remove the remaining water and formic acid) yielded a barium salt which showed no glycerophosphate on chromatography. (Found: N, none detected; P, 7·2; molar ratio inositol:P:glycerol, 1·0:1:0·82. $(C_9H_{12}O_{11}P)_2Ba,4H_2O$ requires P, 7·1 %).

Optical activities of two samples of GPI (free acid) were measured in aqueous solutions containing about 1 mg. of P/ml. and the specific rotations were calculated for the anhydrous compound.

$[\alpha]_D^{20} = -16 \pm 2°$ (sample containing GP)

$[\alpha]_D^{20} = -18 \pm 2°$ (no GP detected)

Hydrolysis of GPI (barium salt) with 0·5N-KOH at 100° for 40 min. completely decomposed the diester. Glycerophosphate and inositol monophosphate were produced and identified by chromatography in (*a*) propan-2-ol–acetic acid–water (3:1:1, by vol.) and (*b*) propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88)–water (5:4:1, by vol.). R_p values relative to GP were as follows: solvent (*a*) IMP, 0·3; GPI, 0·3; GP, 1·0; solvent (*b*) IMP, 0·5; GPI, 1·0; GP, 1·0. A more detailed study of this hydrolysis, and its implications relating to the structure of monophosphoinositide, will be reported (cf. Brown, Hall & Higson, 1958).

GPS was isolated from hydrolysates of ox-brain phosphatidylserine after separation on a column of Dowex 1. Its glycerol content could not be determined by the chromotropic acid method used for GPI, because periodate oxidation of the serine also produced formaldehyde, which caused interference. However, hydrolysis with 3N-HCl at 100° for 90 min. gave only one phosphate, which was identified as glycerophosphate by chromatography in solvents (*a*) and (*b*) (Found: N, 5·2; P, 10·9; N:P molar ratio, 0·95:1·0. Anhydrous GPS requires N, 5·4; P, 11·96 %). Hydrolysis followed by chromatography revealed serine as the only ninhydrin-reactive spot. It was present in equimolar amounts to P. The specific rotation was so small that it could not be accurately measured on the dilute solutions available, but it seemed to be of the order of $+1·9°$ for the free acid in water.

Paper chromatography of glycerylphosphoryl inositol and glycerylphosphoryl serine. For comparison with the work of Dawson (1954), chromatograms of the free acids were prepared in solvents (*a*) and (*b*) above, (*c*) phenol–aq. NH_3 soln. and (*d*) *tert.*-butanol–water (62:38, v/v) containing 10 % (w/v) of trichloroacetic acid. In solvent (*a*) R_p values relative to GP were: GPI, 0·3; GPS, 0·5; GP, 1·0, and in solvent (*b*) both diesters moved to the same position as GP. In phenol, GPI did not move and GPS had an R_p value less than 0·05. With solvent (*d*) R_p values were: GPI, 0·14; GPS, 0·32; GP, 0·61.

Separation of glycerophosphate, inositol monophosphate and inorganic phosphate

A separation of these three compounds on Nalcite SAR resin was reported previously (Hübscher & Hawthorne, 1957). All attempts failed to separate the compounds on Dowex-1 (×2) resin. Only a partial separation of them was obtained with Dowex-2 (×8) resin which is very similar to Nalcite SAR.

Separation of hydrolysis products obtained from freshly prepared liver phosphatides

The livers of three rats were removed as soon as possible after death and homogenized in 5 % trichloroacetic acid. After centrifuging, the washed precipitate was extracted with chloroform–methanol (1:1, v/v). The extracted lipids were subjected to the usual alkaline hydrolysis and the phosphate esters produced were applied in 5 mM-sodium borate to a Dowex-1 column. Much of the phosphate passed right through the column, as would be expected since glycerylphosphoryl choline is a major product of hydrolysis. Elution with the formate–borate solutions described above gave several peaks, two of which corresponded in position with glycerylphosphoryl inositol and glycerylphorphoryl serine. A further peak is thought to be glycerylphosphoryl ethanolamine, since hydrolysis and paper chromatography showed ethanolamine in about equimolar amounts to P, and a fourth remains unidentified. As with brain and pancreas (Hokin & Hokin, 1956) small amounts of glycerophosphate were observed. More detailed studies on liver and other tissues are in progress.

DISCUSSION

The present method can be applied to crude phospholipids freshly prepared from tissues. It should thus be possible to determine the relative amounts of the various glycerophosphatides in animal tissues more accurately than with the other methods so far available. The resin method is also more suitable than paper chromatography for the study of minor unidentified constituents of phospholipid hydrolysates. Little is known yet, for instance, about the hydrolysis product of liver polyglycerophosphatide (McKibbin & Taylor, 1952). Study of ^{32}P uptake into this and other phosphatides should be facilitated by the use of resin columns.

Several workers (e.g. Dawson, 1954; Hokin & Hokin, 1956) have shown that in certain tissues the ^{32}P turnover of inositol phosphatides is much higher than that of the other phospholipids. However, the hydrolysis products on which these results depended were not well characterized. The

present work establishes the structure of the inositol-containing ester from liver phosphatides as glycerylphosphoryl inositol. Previous results suggested that this grouping was present in monophosphoinositide (Faure & Morelec-Coulon, 1954; Hawthorne & Hawthorne, 1955; McKibbin, 1956), but the evidence was not conclusive. It now seems clear that this lipid, which is probably present in most tissues other than nervous tissue, has a structure analogous to that of lecithin, choline being replaced by myoinositol.

It is of some interest that inositol monophosphate, glycerophosphate and inorganic phosphate behaved differently on the three resins used, in spite of the fact that each resin has the same ionic groupings (quaternary ammonium). This suggests that adsorption is determined partly by the structure of the resin matrix, resins with a higher degree of cross-linking giving better separations.

Further hydrolysis of glycerylphosphoryl inositol releases both glycerophosphate and inositol monophosphate. In view of the possibility of 'cyclization' (cf. Brown *et al.* 1958) with the adjacent ring hydroxyls of the inositol, as well as with the β-hydroxyl group of the glycerol, it was at first thought that this diester would be unstable. Its breakdown under the mild alkaline conditions of Dawson's method might then have explained the presence of small amounts of glycerophosphate in hydrolysates of liver and pancreas phospholipids which include monophosphoinositide. The results, however, show that glycerophosphoryl inositol is stable under these conditions and that it differs little in stability from glycerylphosphoryl serine, which possesses only one hydroxyl group vicinal to the phosphate group. The observed glycerophosphate thus cannot arise from monophosphoinositide. This is confirmed by the effects of longer hydrolysis times (30 and 45 min. as compared with Dawson's 15 min.). No further release of glycerophosphate was observed after 15 min. In addition, both Dawson (1954) and Hokin & Hokin (1956) found much higher specific activities for the glycerophosphate than for the inositol ester. This would also exclude a common origin.

The glycerophosphate must therefore arise from phosphatidic acid, as Dawson suggested, or from some phosphatide much less stable than the known glycerophosphatides. The present work does not directly exclude polyglycerophosphatide, but its structure suggests that it would not hydrolyse rapidly and completely to glycerophosphate in 15 min., but rather that a more complex ester would be first formed. Though brain tissue contains diphosphoinositide, not monophosphoinositide, the structures are sufficiently similar for this compound also to be excluded as a source of glycerophosphate.

SUMMARY

1. The mild alkaline hydrolysis of certain phospholipids has been studied.

2. Glycerylphosphoryl inositol and glycerylphosphoryl serine have been separated from one another and from other phosphates, on anion-exchange columns.

3. The two diesters have been isolated by this method and analysed.

4. Glycerylphosphoryl choline is not retained by the columns used.

5. It is suggested that the glycerophosphate observed in hydrolysates of tissue phospholipids cannot arise from any known lipid except phosphatidic acid.

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Electrophoresis Studies of Human Synovial Mucin

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A characteristic property of synovial fluids is the formation of a mucin clot when a weak acid is added. The physical character of the clot may be of some aid in the diagnosis of rheumatoid arthritis (Ropes, Bennett, Cobb, Jacox & Jesser, 1957; Ropes & Bauer, 1953). The clot has also been used as a preliminary step in the isolation of hyaluronic acid (Meyer, Smyth & Dawson, 1939) and its hexosamine content as a measure of the hyaluronic acid (Ogston & Stanier, 1950; Hamerman & Schuster, 1958).

Previous electrophoretic studies of synovial mucin have indicated that the clot is composed of hyaluronic acid and protein (horse mucin; Blix, 1940); hyaluronic acid, albumin and γ-globulin (horse and human mucin; Hesselvik, 1940); and hyaluronic acid or complex, albumin and other proteins (Ogston & Stanier, 1952; Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947). Chemical studies had shown hyaluronic acid and 'globulin' as components (Meyer *et al.* 1939), whereas ultracentrifuge analyses showed the presence of a hyaluronic acid complex and albumin (cattle mucins; Ogston & Stanier, 1950, 1952).

The work described in this paper is a part of a basic study of the composition and significance of human synovial mucin. The composition as indicated by electrophoretic analysis is the principal subject of this paper.

METHODS AND MATERIALS

Sources. Normal and post-mortem synovial fluids comprised the 'normal' group (Platt, Pigman, Holley & Patton, 1956). All were pooled fluids. The post-mortem fluids were obtained from the knee joint at hospital autopsy a few hours after the death of the patient. The normal fluid was a pooled sample obtained from the knee joints of about ten young male volunteers (average age 20 years).

The pathological synovial fluids were divided into two groups: the rheumatoid-arthritis group and a miscellaneous group containing the osteoarthritis and Reiter's-syndrome fluids. No significance was ascribed to the clinical state of the disease.

Preparation of clot. For the preparation of the clot, an equal volume of 5% acetic acid was added to the synovial fluid (pH 4·0). The solution was centrifuged, and the supernatant was decanted and saved. The clot was washed three times with water and the washings were discarded. The clot was stirred in 1% sodium carbonate solution, equal in volume to that of the original synovial fluid sample. Upon stirring, the clot dissolved. The solutions were then prepared for electrophoretic analysis. In several instances, the clots were reprecipitated and redissolved, once as many as five times. All procedures were carried out as rapidly as possible at 4°.

Electrophoretic analysis. For the electrophoretic analyses, the original synovial fluid was diluted with 2 vol. of buffer. Buffer (1 vol.) was added to 2 vol. of the supernatant solution remaining after separation of the clot. Buffer (2 vol.) was added to the dissolved clot solution. The solutions were then dialysed at 4° in Visking cellulose casing against 500 ml. of buffer for 48 hr. The buffer used was sodium diethylbarbiturate (pH 8·6, I 0·1).

The Perkin–Elmer Tiselius-type of electrophoresis apparatus with a 6 ml. cell was used at 0·5°, with a constant current of 9 mA and a time ranging from 13 800 to 20 400 sec. The ascending patterns were used for all mobility determinations, for reasons previously described (Platt *et al.* 1956; Pigman, Patton & Platt, 1957). The concentrations of the various electrophoretic components were calculated from tracings of the electrophoretic patterns enlarged from three to four times. The area for each individual boundary was cut out and weighed and reported in Table 2 as percentage of total refractive-index increment.

Viscosity. Viscosity measurements were made at 30° with Cannon–Manning semi-microviscometers (Cannon Instrument Co., State College, Box 812, Penn., U.S.A.). Water had a flow time of 90 sec. for 0·2 ml. for the usual viscometer; for very viscous fluids, the viscometer had a flow time of 3·7 sec. for 0·23 ml. of water. The measurements were carried out on the diluted fluids as used for electrophoresis. For these measurements, each synovial fluid was diluted to three times its original volume with buffer and dialysed against buffer (pH 8·6, I 0·1). Clots were dissolved as indicated above and the solutions were diluted and dialysed as described for the original fluids.

Ultracentrifugal analysis. Ultracentrifugal analyses were carried out in the Spinco model E analytical ultracentrifuge at 59 780 rev./min. (259 700 g) with the 12 mm. standard analytical cell and the same solutions as for electrophoresis and viscosity measurements.

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RESULTS

Results for the electrophoretic analyses of the mucin clots obtained from human synovial fluids are shown in Table 1. On the basis of the known electrophoretic mobilities of the components of human sera and synovial fluids (Platt *et al.* 1956; Pigman *et al.* 1957), the clot components were identified as indicated in Table 1. All clots contained hyaluronic acid and albumin. α_1-, α_2- and β-Globulins could be detected in some of the clots. The γ-globulin component of serum could not be demonstrated in the clots, and none of them contained more than two identifiable globulin components. The peaks which appeared to be questionable or diffuse are indicated in Table 1 by asterisks.

The π-peak, previously shown to occur in many normal and post-mortem synovial fluids (Platt *et al.* 1956; Pigman *et al.* 1957), was detected in the clot obtained from one of the post-mortem fluids and can be seen in Fig. 1 (*c*), and in the clot obtained from the pooled normal fluid. The π-component was not found in any of the clots obtained from the pathological fluids. A typical pattern of the clot obtained from the pathological fluids is shown in Fig. 1 (*f*).

All of the hyaluronic acid appeared to be precipitated with protein to form the mucin clot, since, in the supernatant, hyaluronic acid could not be detected electrophoretically, (Fig. 1*b*). Under similar conditions, Hamerman & Schuster (1958) reported that all hyaluronic acid was found in the clot.

The clots from post-mortem fluids seemed to be composed almost exclusively of the hyaluronic acid component and albumin (Fig. 1*c*). Globulins, when apparently present, were nearly always questionable. The pooled normal fluid, however, had definite signs of α- and β-globulins. The clots from arthritic fluids showed more regularly the presence of globulins, especially α-globulins.

The amount of clot was always much less for arthritic fluids than for normal and post-mortem fluids. Data on the weights of clots will be given in subsequent papers. Reprecipitation of several clots did not make any noticeable change in their composition. A comparison of the electrophoretic pattern of the original clot (from an arthritic synovial fluid) with that of a twice-reprecipitated and -redissolved clot is shown in Fig. 2. Similar results were obtained after five reprecipitations. The final clots appeared to be very similar to the

Table 1. *Absolute electrophoretic mobilities in the ascending limb for the mucin clot obtained from human synovial fluid*

Values of mobility are given in $-10^5 \times (\text{cm}^2\ \text{v}^{-1}\ \text{sec.}^{-1})$. PM, Post-mortem; RA, rheumatoid arthritis; OA, osteoarthritis; RS, Reiter's syndrome; N, normal. (For details see text.) The last column gives the ratio of viscosity of the clot to that of the fluid from which it was derived under similar conditions of dilution and ionic strength.

Type of synovial fluid	Hyaluronic acid	π*	Albumin	Globulin α_1	Globulin α_2	Globulin β	Viscosity (centipoises)	Viscosity of clot / Viscosity of original fluid
PM	8·6	—	6·4	—	—	—	1·4	0·47
PM	8·8	—	6·6	—	—	—	3·3	0·62
PM	8·9	—	6·6	—	5·0†	—	2·9	0·18
PM	9·6	—	7·4	—	—	—	1·5	—
PM	8·3	7·8	6·5	—	5·0†	3·9†	31	0·58
PM	8·6	—	6·4	—	5·0†	3·9†	6·1	0·10
N	8·9	8·4	6·8	5·6	—	3·9	23	0·18
Average	8·8	8·1	6·7	5·6	5·0	3·9	9·9	—
Range	8·3–9·6	7·8–8·4	6·4–7·4	—	—	—	—	—
RA	9·2	—	6·7	5·4	—	4·0	—	—
RA	9·1	—	6·4	5·8†	4·9†	—	1·7	0·77
RA	9·2	—	6·7	5·2	4·5	—	2·0	0·91
RA	9·0	—	6·7	—	4·5	—	2·3	—
RA	8·8	—	6·5	5·1	—	—	1·7	0·40
RA	8·6	—	6·5	—	4·6	—	—	—
RA	8·7	—	6·4	—	4·6†	3·5	2·0	—
RA	8·7	—	6·6	—	4·8†	3·7†	2·1	—
RA	9·0	—	6·5	5·6	4·8	—	1·7	—
Average	8·9	—	6·6	5·4	4·7	3·7	1·9	—
Range	8·6–9·2	—	6·4–6·7	5·1–5·8	4·5–4·9	3·5–4·0	—	—
OA	8·8	—	6·5	5·1	—	—	1·8	—
OA	8·9	—	6·6	—	4·3	—	1·8	—
RS	8·8	—	6·7	5·2†	—	—	2·6	—

* The π component appears in many normal and post-mortem fluids (see text).
† These peaks appeared diffuse or were questionable.

original, and the composition remained fairly constant, although the absolute amounts had decreased a little as a result of repeated treatment. The apparent shift of albumin to α_1-globulin, shown in Table 2, probably arises from experimental error and the poor resolution of the α_1-peak.

Several electrophoretic patterns were available in which the hyaluronic acid component was sufficiently sharp to allow measurements of the relative areas (and concentrations). The results are shown in Table 2. In the original fluid, the ratio of protein to hyaluronic acid varied widely. However, in the clot obtained from normal, post-mortem and rheumatoid-arthritic synovial fluids, the hyaluronic acid component appeared to account for 12–15% of the total area of the clot pattern. When corrected for the differences in the specific refractive-index increment of hyaluronic acid and protein [Curtain (1955) gives these as 0·00182 for protein and 0·00144 for hyaluronic acid] this range is raised to 15–18%.

In the ultracentrifugal patterns of the normal and post-mortem clots, two components corresponding in sedimentation behaviour to hyaluronic acid and albumin could be detected. Ogston & Stanier (1950) found these components in cattle synovial mucins. The pathological clots contained small amounts of globulin in addition to hyaluronic acid and albumin. The very sharp peak of the hyaluronic acid component appeared to be virtually unchanged in the clot. Hyaluronic acid could not be detected in the supernatant solutions.

The viscosity of the clot was one-tenth to three-fifths of the viscosity of the original synovial fluids in the 'normal' group under comparable conditions of dilution (Table 1). In the pathological group, the viscosity of the dissolved mucin-clot solutions

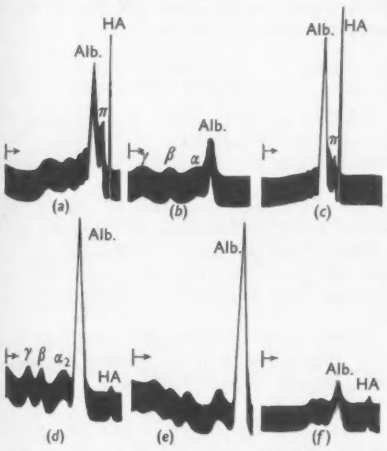

Fig. 1. Ascending electrophoretic patterns of post-mortem (PM) and pathological human synovial fluids, the supernatant and dissolved mucin clot. (*a*) Original PM fluid; (*b*) PM supernatant; (*c*) PM mucin clot; (*d*) original rheumatoid-arthritic (RA) fluid; (*e*) RA supernatant; (*f*) RA mucin clot. Veronal buffer, pH 8·6, *I* 0·1; current, 9 mA; time, 230–340 min. HA, Hyaluronic acid.

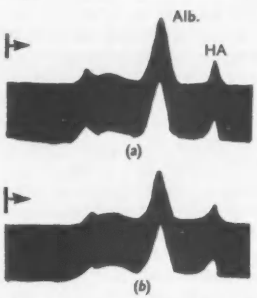

Fig. 2. Ascending electrophoretic patterns of (*a*) the dissolved mucin clot first obtained from rheumatoid-arthritic synovial fluid, and (*b*) the clot obtained after it was reprecipitated and redissolved twice. Veronal buffer, pH 8·6, *I* 0·1; current, 9 mA; time, 225 min. HA, Hyaluronic acid.

Table 2. *Relative refractive increments of components in human synovial fluid and in the mucin clot as measured directly from the electrophoretic patterns*

RA, Rheumatoid arthritis.

	Refractive increment (% of total)						
				Globulin			
Fluid	Hyaluronic acid	π	Albumin	α_1	α_2	β	γ
Original post-mortem fluid	7	3	62	5	5	9	10
Clot (post-mortem)	15	—	75	—	10	—	—
Original fluid (normal)	11	16	44	4	—	16	9
Clot (normal)	15	4	73	6	—	1	—
Original fluid (RA)	2	—	58	1	1	8	30
Clot (RA)	12	—	65	14	9	—	—
Clot (RA), redissolved and repptd. twice	14	—	54	21	11	—	—

ranged from two-fifths to nine-tenths of the value of the original fluid. The supernatant fluids had viscosities corresponding to those of similarly diluted sera.

Since the possibility existed that the treatment with alkali could degrade the hyaluronic acid, the dissolved mucin-clot solutions were kept at 30° and pH 10 for 48 hr. The final viscosity of 16 centipoises was only slightly altered from the original viscosity of 18 centipoises under the same conditions.

DISCUSSION

The electrophoretic analyses of human synovial mucin show clearly that the principal components are hyaluronic acid (or complex) and albumin. As indicated by the patterns and the viscosity of the supernatant fluids, all hyaluronic acid appears in the mucin. These results agree with recent chemical analyses (Hamerman & Schuster, 1958).

Minor amounts of α- or β-globulins may be present in the clots, especially in those from patients with rheumatoid arthritis. The appearance of these globulins may be related to the concentration of all proteins greater in arthritic synovial fluids as compared with normal or post-mortem fluids (Platt *et al.* 1956).

Of the previously described compositions for synovial mucins, these results agree most closely with those of Ropes *et al.* (1947) and of Ogston & Stanier (1950, 1952) for cattle synovial mucin. Ogston & Stanier (1950), however, considered that the hyaluronic acid is present as a 'complex' with protein. This complex is reported to consist of about 75 % of hyaluronic acid and 25 % of protein, mainly α_2-globulin according to Curtain (1955). The complex comprises 45 % of the cattle synovial mucin. The hyaluronic acid would thus compose 34 % of the mucin.

Ropes *et al.* (1947) give the hyaluronic acid as 15 % of cattle mucin, determined by hexosamine analysis. The data given by Hamerman & Schuster (1958) for the nitrogen and hexosamine contents of clots from human post-mortem fluids give an average content of hyaluronic acid of 20·5 % when the hexosamine content of hyaluronic acid is taken as 47·2 %. These values are in close agreement with those for human synovial mucin, 15–18 %, as reported in the present work. Ogston & Stanier's (1950) values, however, are about twice as great, but these workers (1952) point out that the extent of washing may influence the composition.

No evidence for a stable hyaluronic acid–protein complex was obtained in this work. Under the conditions of electrophoresis, the fast component clearly does not comprise 45 % of the total clot. Its mobility also corresponds to those for hyaluronic acids isolated from various sources. The problem is under further study, and the results will be reported later.

Some of the human mucins showed π-peaks in the electrophoretic patterns. However, in several instances this peak was not present in the pattern for the mucin, although shown in that for the original fluid. The significance of this is not known.

The relative constant percentage of apparent hyaluronic acid in three clots from normal subjects, post-mortem and rheumatoid-arthritis patients may be a coincidence, but this finding agrees with the claim of Meyer, Palmer & Smyth (1937) that a definite combining ratio exists. The lack of marked change upon reprecipitation is also in agreement with a fixed ratio.

SUMMARY

1. Human synovial mucin was prepared from normal, post-mortem and pathological fluids, including many from patients with rheumatoid arthritis.

2. Electrophoretic studies at pH 8·6 show that the main constituents are albumin and a fast-moving component. The fast-moving component seems to be hyaluronic acid but might be the complex described by Ogston & Stanier (1950).

3. Particularly in clots from arthritic fluids, α- and β-globulins may be present, though in small amounts. No evidence was obtained for γ-globulins.

4. Analysis of the electrophoretic patterns indicated the presence of 15–18 % of hyaluronic acid in a few clots from normal and pathological fluids. This apparent constancy and the fact that only slight changes were caused by reprecipitation suggest a constant combining ratio of hyaluronic acid and protein.

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The Respiration of Cardiovascular Tissues of the Developing Chick and Adult Fowl

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Little information is available on the oxygen uptake of cardiac tissue, one probable reason for this being the technical difficulty of making accurate observations on such tissue owing to the rapidity with which it deteriorates after removal from the experimental animal (Bernheim & Bernheim, 1944; Webb, Saunders & Thienes, 1949*a*). Values have been reported for the mouse heart by Pearce (1936), for the rat heart by Bertalanffy & Pirozynski (1951), Webb *et al.* (1949*a*) and Nowy & Helmreich (1950), but only a few observations have been made on the chick-embryo heart (Warburg & Kubowitz, 1927).

The present study mainly deals with the oxygen uptake of cardiac tissue in the chicken (*Gallus domesticus*) during incubation, after hatching and in adult cockerels. The results are compared with histological changes observed in the heart during the various stages of development. In addition, further observations were made on the aorta to supplement the previously reported work of Henderson & MacDougall (1956).

MATERIALS AND METHODS

Determination of oxygen uptake

The differential microrespirometer described by Cruickshank (1954) was used for determining the oxygen uptake of tissue slices. Chick embryos ranging in age from 5 to 22 days of incubation, chickens aged 1–16 days after hatching and mature cockerels provided the tissues. In addition to the incubation age, the weight of each embryo minus its yolk sac was recorded. Observations on the external form of the embryo as described by Hamilton (1952) for determining the stage of development were made, but were not so useful for the present purpose as age and weight.

The method was essentially the same as that used by Henderson & MacDougall (1956) in the study of the respiration of chick-embryo arteries. Immediately after removal from the bird, the heart was placed in ice-cold Krebs–Ringer phosphate and cut by free-hand slicing into 0·5 mm. thick fragments. Preliminary experiments showed that slices which were much thicker or thinner than 0·5 mm. gave less than optimum Q_{O_2} values. The speed of transfer of the tissue from the animal, the temperature of the Krebs–Ringer phosphate and the thickness of the slices proved to be factors more critical than in the previous work on arteries.

The medium was that used by Cruickshank (1954), but without streptomycin since prolonged observation was not required. It consisted of 0·5 ml. of horse serum, 0·3 ml. of Krebs–Ringer phosphate and 0·1 ml. of 5% (w/v) glucose solution. The Krebs–Ringer phosphate solution was composed of (as parts by vol.) 0·154 M-NaCl (100); 0·154 M-KCl (4); 0·11 M-$CaCl_2$ (3); 0·154 M-$MgSO_4,7H_2O$ (1). To this was added phosphate buffer (21 parts) prepared by mixing 40 ml. of 0·25 M-Na_2HPO_4 with 2 ml. of N-HCl and diluting this mixture to 100 ml. The tissue slices were placed on lens paper and held floating on the surface of the medium on a platform of tantalum gauze. The gas phase was air, and carbon dioxide was absorbed by filter paper soaked in KOH (2%, w/v). It is possible that higher values for oxygen uptake might have been obtained with oxygen as the gas phase, but this was not used as it was desired to compare the results of the present investigation with those of Henderson & MacDougall (1956). After thermal equilibration for ½ hr. in a water bath at 38°, readings were taken every 10 min. for periods of up to 2 hr., during which time the oxygen uptake was found to be linear for at least the first hour in all cases. After the tissue slices had been dried to constant weight at 100° the uptake of oxygen was calculated as $Q_{O_2} = \mu$l. of O_2/mg. dry wt. of tissue/hr.

Histological methods

Portions of the atrium, apex of the ventricle and aorta adjacent to those used for the respiration studies were examined by a variety of histological methods. Immediately after the death of the animal the tissues were placed in one or more of the following fixatives: Ringer–formol, formol corrosive and Zenker's fluid. After identical processing, paraffin sections (10 μ) were stained by standard methods for connective tissues. To demonstrate collagenous fibres Van Gieson's stain, Mallory's aniline blue and Masson's trichrome (light-green) stain were used. For

elastic tissue, the stains employed were Weigert's, Verhoeff's and Taenzer–Unna's orcein. Two silver-impregnation methods were used for reticulin fibres: Foot's modification of Hortega's silver carbonate method (Edwards, 1950) and Lendrum's (1951) rapid method. Every precaution was taken to ensure that each series of sections received as nearly identical treatment as possible.

Measurement of connective tissue

The optical density of a series of sections of the embryonic ventricle treated by Foot's modification of Hortega's method without counterstain was determined in an EEL Major densitometer (Evans Electroselenium Ltd.). The mean reading for several spots was taken for each section. Owing to the lack of adequate colour in silver-impregnated sections of very young embryos it was impossible to centre such sections accurately over the aperture in the densitometer. Values for the younger embryos therefore could not be obtained.

RESULTS

Oxygen uptake of the ventricle in chick embryos

The Q_{O_2} of the apices of the ventricles from 41 chick embryos was determined in the micro-respirometer. Values were obtained for each day of incubation from 5 to 22 days, and the Q_{O_2} was found to fall from $-24{\cdot}3$ at 5 days to $-4{\cdot}1$ at 22 days. Mean values for each day of incubation are shown in Fig. 1, in which Q_{O_2} is related to age. It will be noted that apart from the initial steepness of the curve between 5 and 9 days, the fall in Q_{O_2} is almost linear. When the Q_{O_2} is related to the weights of the chick embryos as in Fig. 2, the initial steepness of the curve becomes more pronounced owing to the relatively greater rate of growth in the earlier stages of incubation.

Oxygen uptake of the ventricle in chicks after hatching and in adult cockerels

A similar series of observations was made on the oxygen uptake of the apices of the ventricles of 30 chicks ranging in age from the newly hatched to 15 days after hatching. The results are summarized in Fig. 3, from which it will be seen, by comparison with Fig. 1, that there is an initial rise in the Q_{O_2}, becoming evident on the 1st day after hatching, and falling by the third day to the value found on the last day of incubation before hatching. Thereafter there is a continued fall to a value of $-1{\cdot}7$ by the fifteenth day.

Estimations were also made on the oxygen uptake of corresponding portions of the ventricle from 10 cockerels varying in age from 6 months to 3 years. The results, also shown in Fig. 3, show that there is a further reduction of Q_{O_2} with increasing age, to a final value of $-0{\cdot}5$ at 3 years.

Comparison between the oxygen uptake of atrium and ventricle

A similar series of observations to those on the ventricle was carried out on the atria of young hatched chicks and older cockerels. The results, shown in Fig. 3, indicate that the oxygen uptake of atrial tissue is consistently higher than that of the ventricle, but that a similar continuing fall in values occurs as age advances.

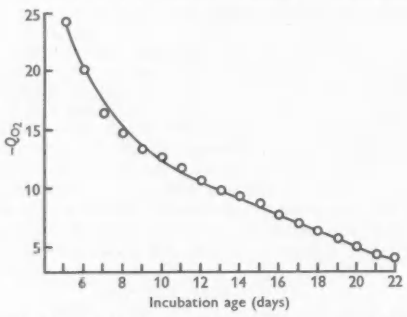

Fig. 1. Relationship between the oxygen uptake of chick-embryo ventricle and the incubation age of the chick. Determinations were carried out at 38° with air as the gas phase. Mean values of two or more determinations are given in all cases, and the range of individual values was within ±5% of the mean value. Although the normal incubation period is 21 days, occasionally an embryo takes longer to hatch. Oxygen uptake (Q_{O_2}) is expressed as μl. of O_2/mg. dry wt. of tissue/hr.

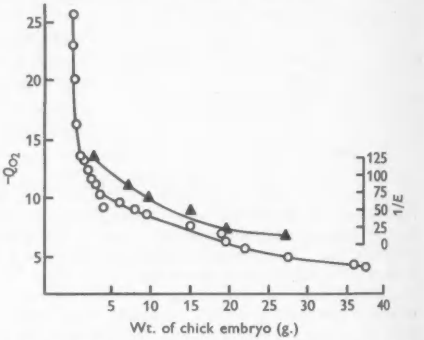

Fig. 2. ○, Relationship between individual values of the oxygen uptake (Q_{O_2}) of chick-embryo ventricle and the weight of the chick. Determinations were carried out at 38° with air as the gas phase. Oxygen uptake is expressed as μl. of O_2/mg. dry wt. of tissue/hr. ▲, Relationship between the reciprocal of the optical density of silver-impregnated sections of chick embryo ventricle ($1/E$) and the weight of the chick.

Oxygen uptake of the aorta

Values were also obtained for the Q_{O_2} of the aorta in young hatched chicks and in cockerels, and were found to be less than the corresponding values for the ventricle. They followed the same general pattern of a gradual fall throughout life, as can be seen in Fig. 3.

Histological observations

The methods used for collagen, elastic tissue and reticulin showed that all three of these fibrous components of the cardiovascular tissues increased with the age of the animal. There were differences between the tissues themselves, however. In the ventricle there is a certain amount of elastic and collagenous material in the subendocardial tissue. Elastic tissue is also present in the walls of the blood vessels found throughout the myocardium. The most striking change with increasing age, however, was found in the proportion of reticular fibres, which were more numerous, thicker and more tortuous in the older hearts.

An increase of elastic tissue with age was more obvious in the atrium than in the ventricle. The development of this tissue appeared to be accelerated in later years.

In the aorta also an increase in the amount of all three connective-tissue fibres was apparent with increasing age. The collagenous and elastic fibres were more affected than the reticular tissue, becoming progressively thicker, longer and more wavy throughout life.

The difference between the fibrous content of any of these tissues in the embryo and in the adult bird was obvious at a glance. The progressive increase began during embryonic life, however, and an attempt to demonstrate this in a quantitative way was made by measuring the optical density of silver-impregnated sections of the chick-embryo ventricle without counterstain, as already described. The values obtained are shown in Fig. 2 as reciprocal readings to facilitate comparison between the results and those of the Q_{O_2} determinations. The similarity in the curves can be seen.

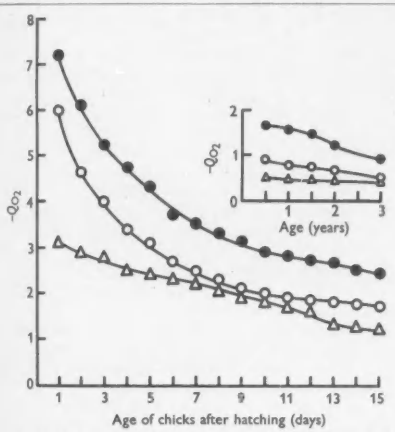

Fig. 3. Relationship between the oxygen uptake (Q_{O_2}) of cardiovascular tissue, in the hatched chick and fowl (inset graph), and the age of the bird (●, Q_{O_2} of atrium; ○, Q_{O_2} of ventricle; △, Q_{O_2} of aorta). Determinations were carried out at 38° with air as the gas phase. Each value represents the mean of two determinations. Oxygen uptake is expressed as μl. of O_2/mg. dry wt. of tissue/hr.

DISCUSSION

As shown by Bernheim & Bernheim (1944) and Webb *et al.* (1949*a*) the extreme sensitivity of the heart to anoxia leads to irreversible changes if any delay occurs between the death of the animal and transfer of tissue to the respirometer. In addition, the large size of cardiac-muscle cells (Shorr, 1942) results in a larger proportion of damaged cells. In the present work, consistent results were obtained by rapid manipulation, and careful slicing of the tissue to the optimum thickness while maintaining it at low temperatures. The inclusion of glucose in the medium, as was shown by Webb, Saunders & Thienes (1949*b*), allowed steady rates of respiration to be maintained for 1–2 hr.

The general pattern of the values for the oxygen uptake of chick-embryo ventricle was similar to that found by Henderson & MacDougall (1956) in chick-embryo arteries and by Maier & Haimovici (1957), who demonstrated a decrease in the oxidative capacity of human aortic tissue with ageing. If the results of the present investigation are compared with those of Henderson & MacDougall (1956), it will be seen that in the younger embryos arterial tissue respires at a slightly higher rate, but that from about the thirteenth day of incubation onwards cardiac tissue is more active, this difference being maintained in the adult bird, whereas the oxygen uptake of atrium, ventricle and aorta continues to decline. The figures for the oxygen uptake of cardiovascular tissue therefore are in agreement with those reported for the intact chick embryo by Philips (1941), and for its homogenate by Albaum, Novikoff & Ogur (1946), and parallel the declining basal metabolism of the fowl described by Mitchell, Card & Haines (1927).

The curious temporary rise in the oxygen uptake of ventricular tissue soon after hatching appears to be an expression of the bird's attempts to adjust itself to a new environment. It was shown by Pembry, Gordon & Warren (1894) that during its life within the egg, the chick is poikilothermic,

except for a few hours preceding hatching, when the transformation to the homoiothermic state occurs. In its changing response to environmental temperatures before and after hatching, the embryo may be compared with the hibernating animal. During hibernation, the response of the animal is typically cold-blooded, but when the animal wakes there is an outburst of metabolic activity and a rapid increase in heat production (Pembry, 1901, 1903). A similar but smaller outburst of metabolic activity occurs in the hatching chick. Apart from this minor fluctuation, more evident in the ventricle than in the aorta, the fall in Q_{O_2} continues and can be demonstrated in the adult cockerel.

The higher values found in the atrium as compared with the ventricle confirm similar results obtained in the rat by Nowy & Helmreich (1950).

As with chick-arterial tissue, the fall in the oxygen uptake of the ventricle with increasing age or weight of the animal appears to be related to an increase in the amount of connective-tissue fibres. In the atrium, as already mentioned, the development of elastic fibres appears to be accelerated in later years, and as can be seen from Fig. 3, the absolute fall in Q_{O_2} of the atrium is greater between 18 months and 3 years than it is with either the ventricle or the aorta.

The available information in regard to other animals is as follows. Lowry, Hastings, Hull & Brown (1942) found that in the rat there was a considerable rise in both the collagen and elastin content of cardiac muscle with increase in age. Bacon (1948) showed that in the myocardium of the mouse the number and thickness of reticular fibres increase with age. Dogliotti (1931) could find no difference in reticular fibres between young and senile human hearts, but Ehrenberg, Winnecken & Biebricher (1954), using quantitative methods, showed a continual increase in the amount of connective tissue in the human heart throughout life.

It is not clear, however, whether the increase of these relatively inert fibres is sufficient to explain the whole of the considerable reduction in the oxygen uptake which was observed. In further work, to be published later, it has been confirmed by chemical and histochemical methods that the increase of these fibres in the cardiovascular tissue of the fowl continues after embryonic life in both heart and aorta, just as the respiration rate falls.

SUMMARY

1. The Q_{O_2} of cardiac- and aortic-tissue slices from chick embryos, hatched chicks and adult cockerels of various ages was determined by means of a microrespirometer.

2. The embryonic ventricles showed Q_{O_2} values ranging from $-24{\cdot}3$ at 5 days to $-4{\cdot}1$ at 22 days of incubation.

3. After hatching there was a further fall in the oxygen uptake of the ventricle, apart from an initial temporary rise. The fall continued into adult life, reaching a Q_{O_2} value of $-0{\cdot}5$ at the age of 3 years.

4. The atria of hatched chicks and adult cockerels showed higher values for Q_{O_2} than the corresponding ventricles. The ventricles in turn gave higher values than the aorta, except in the earlier phases of embryonic life.

5. The continual fall in oxygen uptake of atrium, ventricle and aorta, throughout the life of the animal, appeared to be associated with a gradual increase in the amount of connective-tissue fibres present in the tissue.

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